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(54) Title: ROOT-SPECIFIC PROTEIN INVOLVED IN AUXIN TRANSPORT

(57) Abstract

A root-specific plant gene which encodes an auxin-transport carrier protein that is required for gravitropism; an auxin-transport efflux carrier protein; genetically engineered plants whose genomes comprise heterologous DNA encoding a root-specific auxin efflux carrier protein, or heterologous DNA encoding a portion of an auxin efflux carrier protein sufficient to encode a functional carrier protein and to confer a phenotype characterized by a decreased sensitivity to a herbicide which is an auxin derivative, an auxin analogue or a formulation comprising an auxin transport inhibitor in combination with a second herbicide; and methods useful for identifying molecular targets involved in gravitropic signal transduction, for evaluating the effects of agents on auxin transport and for elucidating the role of gene expression and the molecular mechanism of polar auxin transport.

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ROOT SPECIFIC PROTEIN INVOLVED IN AUXIN TRANSPORT

BACKGROUND OF THE INVENTION

The plant hormone auxin (indole-3-acetic acid (IAA), is involved in plant growth (cell division and cell expansion), morphogenesis (e.g., the differentiation of vascular tissue and lateral or adventitious root formation) and physiological responses to the environment, such as phototropism and gravitropism. Plant tropisms, growth towards or away from a stimulus such as light or gravity, have been ascribed to asymmetric plant growth in which one side of a plant organ elongates to a greater extent than the other, resulting in a curvature toward or away from the stimulus (Darwin, C., *et al.*, (1880) *Power of movements in plants*. John Murray, London; Poff *et al.*, (1994) The physiology of tropisms, In *Arabidopsis,* 639-664, eds. Meyerowitz, E. M., and Somerville, C.R., Cold Spring Harbor Laboratory Press). Root gravitropism (growth in a direction defined by gravity) can be demonstrated by manipulating plants so that they lie horizontal to the surface of the earth (gravistimulation) (Okada, K. and Y. Shimura (1992) *Aust. J. Plant Physiol. 19*: 439-448). Within a short time, the roots curve downward exhibiting a positive gravitropic growth response. Transport studies suggest that IAA is redistributed in response to gravity so that it accumulates along the lower side of the root tip (Young, L.M., *et al.*, (1990) *Plant Physiol. 92*: 792-796). Removal of the root tip abolishes gravitropism; and it is well established that polar auxin transport can be specifically inhibited by synthetic compounds, known as auxin transport inhibitors (Galwelier, L. et al., (1998) Science 282: 2226-2230). Thus, redistribution of IAA in the root tip may be critical to gravitropism (Blancaflor, E.B., *et al.*, (1998) *Plant Physiol. 116*: 213-222). These observations are consistent with earlier views (the Cholodny-Went hypothesis, see Estelle, M., (1996) *Curr Biol. 6*: 1589-91) which suggested that when roots are oriented horizontally (e.g., gravistimulated), IAA accumulates along the lower side of the

elongating zone, resulting in inhibition of cell elongation in those cells while those on top elongate, a process that eventuates in the downward bending of the root.

It is well established that specialized auxin transport systems, comprising auxin influx and efflux activities, exist in several species of plants (Estelle, M., (1996) *Curr Biol. 6*: 1589-91). IAA is thought to be polarly transported, from its point of synthesis in the plant shoot, down to the root (acropetal transport) tip via the vascular system, and then transported up from the root tip to the elongation zone (basipetal transport) where it probably localizes in the epidermis. This polarized cell to cell transport can be explained by the chemiosmotic hypothesis (Goldsmith, M.H.M., (1977) *Ann. Rev. Plant Physiol. 28*: 439-478; Lomax, T.L., *et al.*, (1995) In *Plant Hormones, Physiology, Biochemistry and Molecular Biology,* P.J. Davies, Ed. (Martinus, Nijhoff, Kluwer, Dordrecht, Boston, London, pp 509-530). This model posits that uncharged IAA in the acidic extracellular space enters a cell either by passive diffusion or facilitated transport. Upon entry into the relatively more basic cytosol, IAA dissociates to form IAA^-. The transit of IAA^- (auxin anion) out of a cell and into an adjoining cell is thought to depend on, and be regulated by, an efflux carrier protein (Lomax, T.L., *et al.*, (1995) In Plant Hormones, Kluwer Academic Publishers. Dordrecht, Boston, London; Jacobs, M. and S.F. Gilbert, (1983) *Science. 220*: 1297-1300). Thus, gravitropism could result from the differential activity of an IAA efflux carrier in response to gravity.

Support for the chemiosmotic hypothesis comes from the effects of auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) and N-1-naphtylphtalamic acid (NPA) (Thomson, K.-S., *et al.*, (1973) *Planta (Berl). 109*: 337-352; Katekar, G.F. and A.E. Geissler, (1980) *Plant Physiol. 66*: 1190-1195), which interfere with auxin efflux. (Sussman, M.R. and M.H.M. Goldsmith, (1981) *Planta. 152*: 13-18) Plants grown in the presence of TIBA or NPA are agravitropic (Mulkey, T.J. and M.L. Evans, (1982) *J. Plant Growth Regul. 1*: 259-265; Lee, J.S., *et al.*, (1984) *Planta. 160*: 536-543). Moreover, mutants with altered response to these auxin transport inhibitors have phenotypes consistent with the hypothesis that transport of auxin is critical for the gravitropic response. Mutants resistant to IAA have phenotypes that also support the involvement of IAA in gravitropism. Several auxin resistant mutants are agravitropic

(Estelle, M. and H.J. Klee, (1994) *Arabidopsis* Cold Spring Harbor Laboratory Press). Despite the connection between auxin transport inhibitors and gravitropism, the targets of these inhibitors and the molecules involved in directed auxin transport have not yet been identified.

SUMMARY OF THE INVENTION

Described herein is the isolation and characterization of a plant gene which encodes an auxin-transport-efflux carrier protein that is required for gravitropism. The disclosed protein and gene are targets for regulation of auxin transport in response to the hormones ethylene and auxin (including auxin analogues and auxin derivatives) and the inhibition of auxin transport mediated by synthetic transport inhibitors. Also described are uses of the gene and the encoded protein; mutant forms of the gene and the encoded protein; modified EIR1 nucleic acid molecules; assays which are useful for identifying and characterizing mutant forms of the gene (variant nucleic acid molecules; mutants or alleles) and the encoded protein; methods of altering auxin homeostasis in plant roots; methods of producing genetically engineered plants, such as crop plants and flowering plants, which show greater resistance to herbicides which are auxin derivatives or auxin analogues or formulations comprising an auxin transport inhibitor in combination with a second herbicide than is shown by the corresponding wild type plants; genetically engineered plants with greater resistance to herbicides; seeds, leaves and other plant tissues or parts obtained from such plants; and seeds from which plants with increased herbicide resistance can be produced.

A specific embodiment of the present invention relates to a plant gene, referred to as *EIR*1 (for: Ethylene Insensitive Root) , and its encoded auxin transport (e.g., efflux) carrier protein, EIR1, which is required for gravitropism; assays useful for assessing *EIR1* activity and determining structure/function relationships characteristic of mutagenized alleles of *EIR1*; inhibitors and enhancers of EIR1 protein identified by the assays; methods of increasing transport of (efflux) auxin in plant roots by introducing *EIR1* DNA into the root of a plant, directly or indirectly (e.g., by producing plants from seeds containing exogenous *EIR1* DNA); methods of producing plants which exhibit greater resistance to herbicides (relative to the susceptibility exhibited by

the corresponding wild type plant) which are auxin derivatives or auxin analogues or formulations comprising an auxin transport inhibitor in combination with a second herbicide relative to the susceptibility that is exhibited by the corresponding wild type plants; genetically engineered (e.g., transgenic) plants in which the roots contain and express heterologous DNA, or a portion or fragment thereof which encodes a protein which is involved in auxin transport (e.g. EIR1 DNA, REH1 DNA) wherein the DNA is expressed in the roots as a functional root–specific auxin transport protein; and the transgenic plant exhibits greater resistance (or tolerance) to herbicides which are auxin derivatives or auxin analogues or compositions comprising an auxin transport inhibitor, than the sensitivity exhibited by the corresponding wild type plant; plant tissues or parts obtained from such plant tissues; and seeds from which plants with increased herbicide resistance can be produced. Also the subject of this invention are mutant *eir*1 genes (mutant alleles), the encoded mutant protein and *eir*1 mutant plants, in which the roots are agravitropic and have a reduced sensitivity to ethylene (relative to wild type plants).

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A - 1C are dose response curves of normalized root growth from wild type plants (black circle, Col-O; black square, Ws;) and *eir1* mutants (open circle, *eir1-1*; open square, *eir1-3*) in the presence of 1-aminocyclopropane-1-carboxylic-acid (ACC; the immediate biosynthetic precursor of ethylene), 2,3,5 triiodobenzoic acid (TIBA ;an inhibitor of auxin transport), and napthaleneacetic acid (NAA; an auxin analogue). Root elongation determined at 12 days after germination (DAG) was normalized to root growth on unsupplemented medium (100%). Standard deviations are shown as bars; molarities used are indicated.

Figure 2 is a schematic representation of an *Eco*RI fragment isolated from phage

λ5-3. The bars indicate the 9 exons of *EIR1*. Those segments presumed to be translated are black. Two mutations are indicated beneath the line: Insertion of Ac in *eir1-3* after amino acid 133 and base substitution of the intron/exon junction in *eir1-1*. The grey bar above the line indicates the genomic fragment amplified by inverse PCR

as described herein.. Abbreviations for restriction sites are as follows: RI: *Eco*RI; H: *Hin*DIII; Ba: *Bam*HI; X: *Xba*I; B: *Bcl*I.

Figure 3 shows the alignment of the deduced amino acid sequences of *EIR1* (SEQ ID NO.: 1), the rice homologue *REH1* (SEQ ID NO.: 2) and the two putative *Arabidopsis* homologues *AEH1* (SEQ ID NO.: 3) and *AEH2* (SEQ ID NO.:4). For *EIR1* and *REH1*, ORFs of the cDNAs were deduced. The protein sequences of *AEH1* and *AEH2* were deduced from the genomic sequences by identifying canonical splice donor and acceptor sites. Identical residues are boxed and dashes indicate gaps in the sequence. Black lines correspond to the 10 potential transmembrane domains shared by all 4 proteins. Potential, conserved N-glycosylation sites are typed in bold letters. An arrow indicates the cleavage site of a potential N-terminal signal peptide found for EIR1, REH1 and AEH1.

Figure 4 shows the alignment of the conserved – (top) EIRI, SEQ ID NO.:5; REHI, SEQ ID NO.:6 and C-terminal (bottom) EIRI, SEQ ID NO.: 25; REH1, SEQ ID NO: 26) protein transmembrane domains of EIR1 and REH1 with a number of selected bacterial transporters (mdcF, SEQ ID NO.: 7; livM, SEQ ID NO.: 8; arsB, SEQ ID NO: 9; and sbmA, SEQ ID NO.: 10). Identical residues are boxed. The bold letters represent positions where exchanges are conservative (L, I, V, M; A, S, T; F, W , Y; N, Q; D, E; and K, R) and shared by EIR1 and at least two other sequences. Dashes indicate gaps in the alignment.

Figure 5 is the nucleotide sequence of *EIR1* genomic DNA (SEQ ID NO.: 11), including the promoter.

Figure 6 is the nucleotide sequence of *EIR1* cDNA (SEQ ID NO.: 12) (GenBank accession number AF056026).

Figure 7 is the amino acid sequence of EIR1 protein (SEQ ID NO.: 1).

Figure 8 is the nucleotide sequence of the rice homologue (*REH1)* cDNA (SEQ ID NO.: 13) (GenBank accession number AF056027).

Figure 9 is the amino acid sequence of the rice homologue (REH1) protein (SEQ ID NO. 2).

Figures 10 A and 10 B are graphs demonstrating auxin transport activity in yeast strains *gef*1 and *gef1 EIR1*. The graphs summarize the amount (in percent) of ^{14}C-IAA

remaining in yeast cell samples taken at different points from cells maintained under various assay conditions. The amount of total radioactivity incorporated by the cells was determined in a sample of cells prior to their introduction into the assay. Bars indicate standard deviations derived from 3 parallel samples. Each experiment was performed at least four times. Figure 10 A shows a lack of auxin transport in *gef1* cells assayed in the presence of an external carbon source (2% glucose) in the efflux buffer. Figure 10 B shows the results of an assay performed in the absence of an external carbon source; auxin transport under these conditions depends exclusively on the pre-established membrane potential. Fig. 10 B (*gef1*; *gef1 EIR1*) demonstrates that the expression of *EIR1* in *gef1* yeast results in the retention of about 10 to about 20 percent less ^{14}C-IAA within the cells. The gef1 + CCCP and gef1 EIR1 + CCCP 1 data (Fig. 10B) demonstrate that the inclusion of the protonophore CCCP in the efflux buffer eliminates auxin transport activity.

Figures 11 A and 11 B are graphs comparing the growth of yeast cells expressing either wild type EIR1 or one of three Ser97 negative alleles of EIR1. The conserved amino acid Ser97 of EIR1 was replaced with another amino acid residue, thereby producing three mutants: EIR1-S97G; EIR1-S97A and EIR1-S97E. Figure 11 A shows the growth curve of *gef1* transformed with either EIR1 or one of the Ser97 mutants in Synthetic Complete medium (SC) . Figure 11 B shows the growth curve of either EIR1 or one of the Ser97 mutants in SC supplemented with 200μM 5-fluoro-indole.

DETAILED DESCRIPTION OF THE INVENTION

Described herein is the isolation and characterization of *EIR1*, a plant gene whose function is required for gravitropism. Genetic and physiological analyses of the *EIR1* gene and *eir1* mutants (*eir1-1* , *wav6-52* and *eir1-3)* support a role for *EIR1* involvement in root-specific auxin transport (efflux). Furthermore, the data provided herein indicate that EIR1 protein, which functions as a root-specific auxin efflux carrier, is a target for the regulation of auxin transport. These findings provide molecular evidence for the critical role of auxin transport in gravitropism and provide important targets and reagents useful for elucidating the role of gene expression in gravitropic signal transduction and the molecular mechanism of polar auxin transport.

The present invention relates to an isolated root-specific protein involved in auxin transport, isolated nucleic acid (e.g., DNA, RNA), for example, DNA encoding the protein, mutants of the DNA and altered forms of the encoded root-specific protein, and uses for the proteins and encoding DNA. In a particular embodiment, root-specific DNA designated *EIR1* and modified EIR1 nucleic acids have been isolated and characterized. The EIR1 protein is required for gravitropism and is involved in root-specific auxin transport. In addition, data presented herein supports the role of the EIR1 protein, which functions as an efflux carrier, as a target for regulation of auxin transport by ethylene and synthetic transport inhibitors. Genomic *EIR1* DNA and *EIR1* cDNA nucleotide sequences and the encoded EIR1 protein (amino acid) sequence are presented, as are the nucleotide sequence and amino acid sequences of a rice homologue, designated *REH1* (for: Rice *EIR1* Homologue) and REH1, respectively.

As used herein, the term "DNA encoding a root-specific protein involved in auxin transport" encompasses such DNA from any and all plant types (e.g., mustard plants, corn, rice, wheat and other grains or grasses, other crop plants, flowering plants). Isolated DNA which is the subject of the invention encodes a protein which is involved in root-specific transport, such as EIR1-protein encoding DNA. For example, DNA encoding a protein involved in root-specific auxin transport includes: (a) the sequences presented herein (SEQ ID NOS.: 11-13) and portions of any of those sequences, provided that they encode a functional root-specific auxin transport carrier protein; (b) DNA which, due to degeneracy of the genetic code, encodes EIR1 protein of the present invention (e.g., EIR1 protein having the amino acid sequence of SEQ ID NOS.: 1, 2, 5 or 6); (c) DNA which hybridizes under high stringency conditions to the complement of any DNA of (a) or (b) and; (d) DNA which is from Arabidopsis or from a plant species other than Arabidopsis which is sufficiently similar in sequence to DNA of (a), (b) or (c) to encode a root-specific protein involved in auxin transport (e.g., as demonstrated by the assay described herein). Homologous DNA can be identified by substantial nucleic acid sequence homology to an EIR1 nucleic acid. For example, homologous DNA can be identified based upon overall nucleic acid sequence homology with the EIR1 DNA sequence disclosed herein, allowing for the degeneracy of the genetic code and codon bias in different species of plants, and on the requirement that homologous sequences encode a functional root-specific auxin transport (efflux)

carrier protein. For example, the overall homology of the nucleotide sequence is preferably greater than about 40%, preferably greater than 60% , still more preferably greater than about 80% and most preferably greater than 90% homologous. Thus, the invention also comprises the use of the disclosed nucleic acid sequences, or portions thereof, as probes and primers for the identification and isolation of homologous sequences from other species of plants.

DNA of the present invention also includes coding or noncoding DNA which is the complement of any of the DNA of (a) - (d) and portions (or fragments) thereof which are of sufficient length (e.g., at least four to six nucleotides) to hybridize to complementary DNA and remain hybridized (e.g., in order that hybridization can be detected, such as for diagnostic or assay purposes). Such fragments also include those which hybridize to characteristic portions of the DNA of the present invention (e.g., to a characteristic portion of DNA of SEQ ID NOS.: 11, 12 or 13). The complement of DNA encoding a root-specific protein of the present invention is also a subject of this invention. For example, DNA complementary to all or a portion of EIR1 protein encoding DNA, such as DNA of SEQ ID NO.11 or SEQ ID NO. 12, is the subject of this invention. Such complementary DNA is useful as probes and primers, for example, in hybridization and amplification (e.g., PCR) reactions.

As used herein, the term "modified EIR1 nucleic acid" refers to a variant EIR1 nucleic acid molecule which includes addition, substitution, insertion or deletion of one or more nucleotide(s), thereby producing a modified nucleotide sequence. As used herein, the term "nucleic acid" encompasses DNA (genomic and cDNA), RNA and analogues (e.g., comprising base analogues such as inosine) thereof. The "modified EIR1 nucleic acid" can embody either a naturally occurring allelic variant or a synthetically produced sequence. For example, the disclosed naturally occurring (e.g., wild type) nucleic acid isolated from *Arabidopsis thaliana* can be used as a precursor nucleic acid molecule which can be modified by standard techniques that are well-known to those of skill in the art to produce a synthetic variant. For example, site-directed mutagenesis or cassette-mutagenesis can be used to substitute one or more nucleotides.

Promoters and other regulatory sequences (e.g., cis acting elements and/or transcriptional enhancers) of DNA encoding a root-specific auxin transport protein are

also the subject of this invention, as are their use in vectors and expression systems designed to direct the tissue-preferential transcription of foreign (e.g., heterologous) genes operably linked thereto, in the roots of plants.

The isolate nucleic acid which is the subject of the invention can be obtained from a plant as it occurs in nature, or can be produced by synthetic (e.g., chemical) methods or recombinant methods. Also included herein are mutant genes, such as the mutant gene designated *eir1*-3 which is present in an agravitropic mutation. This mutated gene and the agravitropic mutation are useful to study the pathway of which *EIR1* is a component.

The isolated root-specific proteins involved in auxin transport and allelic variants thereof which are the subject of the invention include the encoded protein products of the DNA sequences disclosed herein and functional portions and fragments thereof. In particular embodiments the invention comprises proteins having the amino acid sequence comprising SEQ ID NOS.: 1 and 2.

Genetically engineered plants (e.g., transgenic, transformed plants expressing heterologous DNA episomally, transiently, or stably integrated into plant nuclear DNA), plant tissues and seeds characterized by an increased resistance (or tolerance) to the effects of herbicides which are auxin derivatives, auxin analogues, or an herbicidal formulation comprising at least one auxin transport inhibitor applied in combination with at least one additional herbicide, relative to the corresponding wild type plants are also the subject of this invention. More specifically, the invention relates to plants, plant tissues , and seeds which are resistant to growth inhibition by an herbicide (which is an auxin derivative or an auxin analogue), or an herbicidal composition (which includes an auxin, analogue derivative, auxin analogue or auxin transport inhibitor), at concentrations which normally inhibit the growth of those plants, plant tissues or seeds. In one embodiment, the present invention relates to a method of producing a transgenic plant characterized by altered auxin homeostasis. The method comprises introducing DNA encoding a root-specific auxin transport carrier protein into a plant cell under conditions in which the DNA is expressed, thereby producing a transformed plant cell; and producing a transgenic plant from the resulting transformed cell. Transgenic (genetically engineered) plants can be produced using DNA described herein and methods known to those of skill in the art. For example, DNA encoding a

root-specific auxin transport protein can be introduced into plants or plant tissues (e.g., roots) or seeds by transformation (e.g., transfection or transduction) using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, protoplast fusion, electroporation or bombardment (e.g., microprojectile bombardment) with nucleic acid-coated particles.

As used herein, the term "herbicide" refers to compounds which combat or control undesired plant growth. The term "auxin transport inhibitor" refers to compounds which act by inhibiting the transmembrane movement (e.g., transport) of auxin which accumulates in cells as a result of polar auxin transport and affects plant growth. Thus, as used herein, auxin transport inhibitors are themselves herbicides. The observation that auxin transport inhibitors are usually highly active herbicides is consistent with this usage. As used herein, the terms "resistance" and "tolerance" refer to the sensitivity of a plant to the toxic effects of an herbicide, such that a genetically engineered plant, whose genome comprises a nucleotide sequence encoding a root-specific heterologous auxin transport carrier protein is resistant to an herbicide. Genetically engineered plants (transgenic plants) of the present invention include, but are not limited to, vascular plants, including gymnosperms and agronomically important plant crops, such as rice, wheat, barley, rye, corn, soybeans, canola, sunflower, sorghum, sugarcane, fruits (oranges, grapefruit, lemons, limes, apples, pears, melons, plums, cherries, peaches, apricots, strawberries, grapes, raspberries, pineapples, bananas), vegetables (potatoes, carrots, sweet potatoes, beans, peas, lettuce, cabbage, cauliflower, broccoli, turnip, radishes, spinach, onions, garlic, peppers, pumpkins) and angiosperms or flowering plants, both monocots and dicots.

In one embodiment, plants with greater resistance are genetically engineered plants whose root cells comprise heterologous DNA which encodes a protein involved in auxin transport (e.g., *EIR1* DNA, *REH1* DNA) which is expressed as a functional root-specific auxin transport (e.g., efflux) protein. The corresponding wild type plant differs from the genetically engineered plant in that the wild type plant has not been altered to comprise the heterologous DNA present in the genetically engineered plant. In one embodiment, the heterologous DNA which encodes an auxin specific efflux carrier protein is constitutively expressed in a tissue-specific (e.g., root tissue) fashion and the expression trait and resulting phenotype is stably transmitted (sexually and

somatically) to progeny cells. In a second embodiment, the invention comprises transgenic plants, the cells of which comprise heterologous DNA stably integrated into the plant nuclear DNA. In an alternative embodiment, the expression of the heterologous DNA encoding an auxin specific efflux carrier is inducible.

In a second embodiment transgenic plants characterized by an altered auxin homeostasis exhibit a distinctive phenotype, attributed to increased auxin efflux, such as an increased number of lateral or adventitious roots. Such plants may also be further characterized by an increased auxin transport rate relative to the auxin transport rate of a corresponding wild type plant.

As used herein, the term "heterologous DNA" means DNA isolated from a source other than the plant, or plant cell, in which it is expressed (e.g., from a source other than the cell into which it is introduced or in which it is present as a result of having been introduced into a precursor cell, such as seeds or plant tissue from which a plant develops or seeds or plant tissue obtained from a genetically engineered plant). The heterologous DNA can be from the same plant type (e.g., *Arabidopsis* DNA introduced into *Arabidopsis*) or from a different plant type (e.g., *Arabidopsis* DNA introduced into corn, wheat, rice or other plant type, rice DNA introduced into corn, wheat or other plant type). Heterologous DNA can be used, for example, to avoid or reduce the silencing or inactivation to which the endogenous gene or its encoded protein (e.g., post-translational modification) can be subjected. As a result of the presence and expression of heterologous DNA encoding a root-specific auxin transport protein in roots of a genetically engineered plant, auxin transport (efflux) is enhanced and the plant exhibits enhanced resistance to auxin derivatives auxin analogues or formulations comprising an auxin transport inhibitor in combination with a second herbicide. For example, plants (e.g., crop plants, flowering plants, gymnosperms) which are genetically engineered to include or are produced from seeds, plant tissues, or plant parts which include *EIR1* or *REH1* DNA can be produced to provide genetically engineered plants with enhanced herbicide resistance. Plant part is meant to include any portion of a plant from which a regenerated plant can be produced.

Plants which show increased auxin transport and/or enhanced root tissue growth and/or differentiation (compared to the corresponding wild type plants) resulting from altered auxin homeostasis are also the subject of this invention. More specifically, the

invention also comprises genetically engineered plants comprising a heterologous DNA sequence encoding a root-specific protein involved in auxin transport, wherein the genetically engineered plant exhibits a distinctive phenotype, relative to the phenotype of an isogenic plant which does not comprise a heterologous DNA encoding a protein involved in root-specific auxin transport, attributed to the effects of altered auxin homeostasis. For example, transgenic plants characterized by a phenotype comprising an increased number of lateral or adventitious roots.

Also described are alleles of EIR1, in which the conserved residue Ser97 of EIR1 is replaced with another amino acid. Three alleles, EIR1-S97G, EIR1-S97A and EIR1-S97E, were created and characterized, as described in Example 10. These alleles were expressed in diploid yeast strains, defective for the *gef1* gene, under the control of the ADH-promoter. The strains were tested in a filter assay carried out with either 5-fluoro-indole or 5-fluoro-indole acetic acid. The strains exhibited a hypersensitivity to these compounds.

Also described herein is an assay for assessing agents (compounds and molecules) for their effects on auxin transport. As described in the examples, an assay is available in which auxin transport is assessed in yeast by measuring transport of detectably labeled (e.g., radiolabeled) auxin. This assay is useful to determine whether an agent inhibits or enhances the activity of EIR1 protein and, as a result, inhibits or enhances auxin transport. The auxin transport assay can be used for example to characterize EIR1 alleles identified by their ability to confer an altered growth phenotype. For example, one would expect to find an increased auxin transport rate associated with an allele which confers significantly increased resistance of *gef1* yeast cells to fluoroindolics. The yeast cell-based overexpression model disclosed herein provides a functional assay useful for assessing structure/function relationships in isolated DNA molecules and mutated EIR1 sequences encoding auxin transport proteins and their variants. In adition the yeast cell-based overexpression model can be used to identify an allele (mutant) of EIR1 which confers altered auxin-mediated responses in a plant. Briefly the overexpression assay comprises: introducing a mutated EIR1 nucleic acid into yeast cells, thereby producing transformed yeast cells; contacting the transformed yeast cells with a fluorinated indolic compound under assay conditions which favor the diffusion of the compound into the yeast cells; determining the growth phenotype of the cells;

and comparing the growth phenotype of the transformed cells to the growth phenotype of wild type cells, wherein detection of an altered growth phenotype in the transformed cells relative to the growth phenotype of wild type cells is indicative of a nucleic acid which is an allele that results in altered auxin-mediated responses in a plant. The altered growth phenotype observed in the overexpression assay can be either an increased tolerance or an increased sensitivity to concentrations of the fluorinated indolic compounds, relative to the sensitivity of wild type cells. Diploid yeast cells which are defective for the GEF1 gene, and therefore have an altered ion hemostasis are particularly useful for the establishment of an overexpression assay. The overexpression assay is useful, for example, to identify mutant nucleotide sequences, produced by random mutagenesis of wild-type DNA sequences encoding auxin transport proteins which exhibit altered growth phenotypes (either enhanced or decreased sensitivity) to fluorinated indolic compounds. Yeast strains exhibiting altered growth phenotypes (tolerance or increased sensitivity) comprise mutated DNA sequences which upon introduction into a transgenic plant will alter auxin homestasis and auxin-mediated responses such as growth, morphogenesis (lateral or adventitious root formation) and tropisms (gravitropism). The present invention also comprises transgenic plants comprising mutant *EIR1* alleles identified in the yeast cell-based overexpression assay.

The sequences (nucleotide and amino acid) and topology of EIR1, its homology to several bacterial carrier proteins and its function establish that EIR1 functions as a root-specific auxin transport (efflux) carrier protein involved in gravitropism

The present invention is illustrated by the following examples, which are not intended to be limiting in any way. Further, all references referred to herein are expressly incorporated by reference.

EXAMPLES

METHODS AND MATERIALS

The following methods and materials were used in the work described herein, particularly in the examples:

Plant Strains and Growth Conditions

Plants were grown aseptically on unsupplemented PNA (Plant Nutrient Agar) without sucrose. (Haughn, G.W. and C. Somerville, (1986) *Mol. Gen. Genet. 204*: 430-434). Growth responses were tested by adding various supplements to the medium as indicated. Plates were wrapped in gas-permeable surgical tape and kept under continuos illumination. For gravitropic response experiments, plates were kept in a vertical position. For the "root waving assay" plates were kept at an angle of about 30 degrees. Root elongation was assayed at 10-12 days after germination (DAG). Formation of lateral roots was compared by counting lateral roots on both wild type and mutant plants grown under conditions described herein.

Seed stocks for *eir1-1* and *eto3-1* were obtained from the Arabidopsis Biological Resource Center at OSU, Columbus, OH). *ctr1-1* was a kind gift from J. Hua at Caltech, Pasadena, CA. *agr1-52* was obtained from K. Okada, National Institute for Basic Biology, Okazaki, Japan. *PIG4*::GUS was a kind gift from J. Normanly, University of Massachusetts, Amherst, MA. Transposon line B222 was obtained from DNA Plant Technology Corporation, Oakland, CA.

Inverse PCR Cloning and Structural Analysis of *EIR1*

Genomic DNA was prepared according to a protocol from Quiagen. After grinding the frozen tissue, the resulting powder was incubated at 74°C for 20 minutes in lysis buffer (100 mM Tris/HCl pH 9.5, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB, 1% PEG 8000). After extraction with an equal amount of chloroform, DNA was precipitated with isopropanol. After resuspension in 1 M NaCl and treatment with RNase A, the DNA was loaded onto equilibrated Quiagen columns and purified according to the manufacturer's instructions. DNA extracted from the Ac line *B222* and *eir1-3* was digested with *EcoR*I and *Bcl*I. The ends of the DNA were made blunt with Klenow fragment. This DNA was religated and used for inverse PCR performed with oligonucleotides CCTCGGGTTCGAAATCG (SEQ ID NO.: 14) and GGGGAAGAACTAATGAAGTGTG (SEQ ID NO.: 15). After 40 cycles of amplification at 60°C annealing temperature, the products were separated on 1% agarose gels. A fragment specific for *eir1-3* DNA was cloned into pGEMT (Promega) to give pGsac1 and used for Southern hybridization on *eir1-3* and wild type DNA.

Phage genomic and cDNA libraries of *A. thaliana* (Kieber, J.J., *et al.*, (1993) *Cell 72*: 427-441) were probed with pGsac1 using standard techniques. (Ausubel, F.M., *et al.*, (1987) *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc). Genomic clone λ5-3, which hybridized to pGsac1, was subcloned into pBluescriptII (Stratagene) to give pB5-3. The sequence of an *Eco*RI fragment approximately 8kb in length was determined on an ABI Automated DNA sequencer. For sequence analysis of *eir1-1*, the coding region of this allele and its corresponding wild type (Col-O) were amplified with PCR. The point mutation in *eir1-1* was confirmed by subsequent PCR amplification of sequences covering the mutation.

Two full-length cDNA clones subcloned into pBSII (pBc5-2 and pBc6-1) were completely sequenced. The rice EST (D25054) homologous to *EIR1* was obtained from MAFF DNA Bank at the National Institute of Agrobiological Resources (NIAR), Japan.

Sequence comparisons with Database entries were performed using Gapped BLAST and PSI-BLAST algorithms. (Altschul, S.F., *et al.*, (1997) *Nucleic Acids Res. 25*: 3389-40.2) Multiple alignments and structural predictions were performed using the algorithms at BCM Search Launcher.

Complementation of *eir1-1* in Transgenic Plants

An *Eco*RI fragment of the genomic clone pB5-3, which carries the entire coding region and more than 2kb of upstream sequences of the *EIR1* gene was subcloned into pBIBhyg (Becker, D., (1990) *Nucl. Acids Res. 18*: 203). The resulting T-DNA vector pBRL was transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation, and used for subsequent vacuum infiltration of *eir1-1* plants. (Bechtold, N., *et al.*, (1993) *C.R. Acad. Sci. Paris, Sciences de la vie/Life sciences 316*: 1194-1199) Correct integration of the full-length transgene was confirmed on Southern blots.

RNA Template-Specific Polymerase Chain Reaction (RS-PCR)

For expression analysis, total RNA from tissue of sterile grown plants was isolated. (Niyogi, K.K. and G.R. Fink, (1992) *Plant Cell. 4*: 721-33) Vegetative tissue isolated

from plants 15 DAG was used. Flower-specific RNA was isolated at approximately 20 DAG and silique-specific RNA at about 25 DAG. polyA$^+$ RNA was isolated with the polyATract kit from Promega. About 50 ng of polyA$^+$ RNA of each tissue was used for RNA Template-Specific PCR (RS-PCR).

RS-PCR with slight modifications was performed as described by (Shuldiner, A.R., *et al.*, (1993) *In: Methods in Molecular Biology: PCR Protocols: Current Methods and Applications* Human Press Inc. Totowa, NJ). Oligonucleotides GAACATCGATGACCAAGCTTAGGTATCGATAGCCCCACGGAACTCAAA (SEQ ID NO.: 16) (underlined bases are complementary to nucleotides 454 to 470 of the *EIR1* coding region) and CTTATACGGATATCCTGGCAATTCGGACTTGTTAG CTTTAGGGTTAA (SEQ ID NO.: 17 (underlined bases are complementary to nucleotides 335 to 351 of *ACT2* coding region) were added to polyA$^+$ RNA to a final concentration of 2 μM in a volume of 10 μl. The tubes were placed at 65°C for 10 minutes and allowed to cool down to 37°C. First strand cDNA synthesis was performed using Gibco BRL AMV Reverse Transcriptase. Primer pairs GAACATCGATGACC AAGCTTAGGTATCGATA (SEQ ID NO.: 18) and GGCAAAGACATGTACGATGT TTTAGCGG (SEQ ID NO.: 19) (bases 10 to 37 of *EIR1* coding region) or CTTATACGGATATCCTGGCAATTCGGACTT (SEQ ID NO.: 20) and GTCTGTGACAATGGAACTGGAATG (SEQ ID NO.: 21) (bases 31 to 54 of *ACT2* coding region) were used in a standard PCR for 30 cycles with 40 seconds at 94°C, 40 seconds at 60°C and 1 minute at 72°C. For *EIR1*, 1/100 of this reaction was used for reamplification under the same conditions. ^{32}P-end labeled oligonucleotide GTGAAAAGAGCGTTAT CATCCATTCTAG (SEQ ID NO.: 22) (complementary to bases 292 to 319 of *EIR1* coding region) allowed verification of the identity of the *EIR1*-specific band on a Southern blot.

Preparation and Microscopic Analysis of Roots

Whole plants were incubated twice in methanol:glacial acetic acid (3:1) and rinsed several times in PBT (130 mM NaCl, 10 mM sodium phosphate pH 7.0, 0.1% Tween 20). Roots were then mounted onto microscope slides into clearing solution (stock solution: 8 g chloralhydrate in 2.5 ml 20% glycerol). After 10 minutes, roots were viewed under a Zeiss microscope using Nomarski Optics. Dark field photographs of

live plants were made using a Wild M5-A microscope. These images were used for determination of root cell length. GUS stainings were performed as described. (Lehman, A., *et al.*, (1996) *Cell. 85*: 183-94) Images were recorded on Kodak Ektachrome 160T film and processed using Adobe Photoshop.

Complementation Analysis and Construction of Double Mutants

For complementation analysis of the three putative *eir1* alleles, *eir1-3/eir1-3* plants (*eir1-3* still contains the Ac-donor T-DNA-construct conferring hygromycin resistance) were crossed into plants homozygous for either *eir1-1* or *wav6-52*.

Heterozygous F1 plants (*eir1-3/wav6-52 and eir1-3/eir1-1*) identified as resistant to hygromycin were defective in root gravitropism, giving evidence for the allelism of the three mutants analyzed. F2 plants derived from each of the F1 heterozygotes were all Eir1 $^{-}$ whereas the hygromycin resistance marker segregated as a single, dominant locus. Double mutants (e.g. *ein2-1/ein2-1 eir1-1/eir1-1*) were derived from crosses of homozygous single mutant lines and scored for segregation in the F2 generation of the initial crosses. Double mutant candidates were backcrossed into their two parental single mutant lines and their genotype verified by complementation with parental testers. For *eir1-1 alf1-1* double mutants, we used *eir1-1/eir1-1* plants for pollination of *alf1-1/ALF1* heterozygotes. F2 seeds were scored for segregation of Eir1 $^{-}$ and Alf1 $^{-}$ phenotypes. The double mutant was verified by segregation of the aerial Alf1 $^{-}$ phenotype in Eir1 $^{-}$ F3 plants derived from the initial cross.

Auxin Transport Assay

Yeast strains transformed with pAD-EI and pAD4M (Luschnig et al., 1998) were grown to an O.D. 600 of 0.8 to 0.9. Cells were pelleted and an aliquot corresponding to 15ml starting culture was washed in 10mM Na Citrate buffer pH 4.5. The pellet was resuspended in 1ml of 10mM Na Citrate (pH 4.5) supplemented with 1mM IAA (final concentration) and 2.5 micro Ci ^{14}C-IAA (Sigma).

The cells were allowed to incorporate the tracer for 10 or 20 minutes. The cells were subsequently washed on MF-filters (Millipore) on a multifiltration unit, and

resuspended in Synthetic Complete (SC)-medium adjusted to pH 4.0 with HCl. Aliquots of the suspension were dropped onto MF-filters and washed twice with SC-medium
(pH 4.0). The dry filters were transferred into Scintilation Cocktail and radioactivity was determined in a Scintilation Counter. Each experiment was performed for at least 4 times. The radioactivity remaining in the cells is expressed as percentage of total radioactivity present in the washed pellet prior to the efflux assay. Each time point was determined by 3 parallel samples. For the experiments performed in the presence of glucose, 2% (w/v) glucose was added to the efflux buffer. Similarly, for assays performed in the presence of the protonophor CCCP 0.5mM (final concentration) of CCCP were added to the efflux buffer prior to the efflux experiment.

Yeast Manipulations and Constructs

All experiments were carried out in W303 (a/α *ura3-1 can1-100 leu2-3, 112 trp1-1 his3-11, 15*). Plasmid pRG52 was used for disruption of *GEF1*, For analysis of *CLC-0* the vector PRS1024 was used (for more details, see Gaxiola, R.A., *et al.*, (1998) *Proc. Natl. Acad. Sci. USA 95*: 4046-4050).

Yeast strains were grown over night at 30 °C in Synthetic Complete medium (SC) and approximately $2x10^6$ cells were plated onto SC plates. Solutions of inhibitors used in the filter growth assays were spotted onto Schleicher & Schuell Filter Paper #740. After they dried, the filters were transferred onto the yeast plates, which then were incubated at 25%C in the dark for two to five days. After that, yeast growth was monitored and documented.

For expression of *EIR1* in *S cerevisiae* the insert of pBc5-2 was cloned into pAD4M (described in Ballester *et al.*, (1989) *Cell, 59*: 681-686) to give pAD-E1. A frameshift mutation in *EIR1* was obtained by filling in the internal *Hin*dIII site resulting in a nonsense mutation after codon 178 (plasmid pADE1-H). For construction of the HA-tagged version of *EIR1*, we used primers
GGGTCTAGAGTACTCTACTACGTTCTTTTGGGGCTTT
ACCCATACGATGGTCCTGAC (SEQ ID NO.: 23) and
GGGTCTAGAGTCGACGCA CTGAGCAGCGTAAT (SEQ ID NO.: 24) for PCR

amplification of a fragment encoding 3 copies of the HA-epitope. The PCR product was ligated into pAD-E1 resulting in pAD-E1HA coding for a protein with the 3xHA-tag fused to the authentic C-terminus of EIR1. Immunostaining of the tagged protein in haploid and diploid cells was performed as described by Gaxiola, R.A., *et al.*, (1998) *Proc. Natl. Acad. Sci. USA 95*: 4046-4050. Cells were viewed by using charge-coupled device microscopy and sectioned by using SCANALYTICS (Billerica, MA).

EXAMPLE 1

Isolation and phenotypic characterization of *eir1-3*

An agravitropic mutant (e.g., a plant whose roots do not respond to gravistimulation) was isolated from the Ac transposon pool B222-24 (Keller, J., *et al.*, (1992) *Genetics, 131*: 449-59). This agravitropism segregated as if it resulted from a mutation in a single gene. A comparison of DNA isolated from the mutant transposon-tagged line B222-24 with the untransposed parental line B222 on Southern blots revealed that the mutant contained an additional copy of the transposon. This extra Ac element cosegregated with the mutant phenotype, suggesting that the mutation, designated *eir1-3* was caused by the insertion of the transposon element.

This agravitropic mutation, *eir1-3*, is allelic to two previously described mutations, *wav6-52* (allelic with *agr1*), which was isolated as an agravitrophic mutant (Bell, C.J. and P.E. Maher, (1990) *Mol. Gen. Genet. 220*:289-293) and *eir1-1* , which was isolated as an ethylene insensitive mutant (Roman, G., *et al.*, (1995) *Genetics. 139*: 1393-1409). The new mutation, *eir1-3*, fails to complement *wav6-52*, and *eir1-1* showing that all three are alleles of *EIR1*. All three mutants have similar phenotypes with the severity of the mutant phenotype in the order *eir1-3 = eir1-1 > wav6-52*.

eir1 mutant roots do not respond to gravity when germinated and grown on agar plates oriented vertically. Instead, *eir1* roots grow in random directions, whereas *EIR1* roots grow downward. If the seedlings are reoriented so that the roots are now parallel to the surface of the earth, after 24 hours, the roots of wild type reorient downward (roughly 90%), whereas roots of *eir1* fail to reorient their growth.

These severe defects in gravitropism appear to be restricted to the root, as the hypocotyl in all three *eir1* mutant strains tested, still reorients when germinated in the dark. In another assay, seedlings were kept on 2% agar plates that were tilted vertically at an angle of less than 90°. Under these conditions, *EIR1* roots do not penetrate the agar but grow on the surface in a wavy pattern, that is caused by reversible turns of the root tip. (Okada, K. and Y. Shimura (1990) *Science 250*: 274-276) By contrast, *eir1* roots exhibit a roughly linear growth pattern interrupted by random turns. When wild type seeds are germinated on plates whose surface is parallel to the surface of the earth, they enter the agar and form a characteristic array of almost concentric curls. (Garbers, C., *et al.*, (1996) *Arabidopsis. EMBO J. 15*: 2215-2124) However, *eir1* mutant roots failed to curl on the bottom of the plate and grew out in irregular patterns.

Root growth of *eir1* mutant plants is less sensitive to ethylene than that of the wild type, suggesting an involvement of ethylene in the regulation of root tropic responses. *eir1* roots have a phenotype that is similar to *EIR1* roots grown in the presence of NPA and TIBA, inhibitors of auxin transport that block cell elongation (Sussman, M.R. and M.H.M. Goldsmith, (1981) *Planta 152*: 13-18). Moreover, *eir1* root elongation was much more resistant than *EIR1* to NPA and TIBA (Figures 1A - 1C). By contrast, these auxin transport inhibitors inhibit lateral root formation to the same extent in both wild type and *eir1* mutants. Also, *eir1* root growth is more resistant than wild type to 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate biosynthetic precursor of the growth regulator ethylene (Figure 1A). However, the root growth inhibition of *eir1* mutants is no different from *EIR1* with respect to other growth regulators (abscissic acid, gibberellic acid, kinetin), the auxin-analogue NAA (-napthaleneacetic acid) (Figure 1C), and 2,4-D (2,4-dichloro-phenoxyacetic acid).

The *eir1* mutants have longer roots than wild type plants (Table 1), which could be due to an increased rate of cell division and/or to greater elongation of individual root cells. Direct measurement showed that *eir1-3* root cells were longer than wild type cells (Table 1). However, it is possible that increased cell division contributes to the increased length as well.

Table 1 Root Growth and Cell Elongation

Strain	average root length[a]	average cell length[b]
Ws	79 ± 7	102.9 ± 12.4
eir1-3	97 ± 11	135.9 ± 15.3

[a] Length of primary roots was determined at approximately 12 DAG;

[b] Elongation of 35-40 young trichoblasts was determined on images. Root lengths are indicated in mm, cell length in µm.

EXAMPLE 2

Cloning of *EIR1*

The *eir1-3* allele was cloned using an inverse Polymerase Chain Reaction (PCR) approach. A 600 bp fragment amplified from *eir1-3* DNA hybridized to the additional band caused by the Ac transposon element insert in *eir1-3*. This subcloned fragment was used to screen an *A. thaliana* genomic phage library. Three genomic clones of the putative *EIR1* gene (λ5-3, λ6-1 and λ6-3) had the same restriction pattern. The subcloned insert of λ5-3 was used for screening cDNA libraries. Eight hybridizing phage clones were isolated from approximately $5x10^5$ plaques screened. These clones all show similar restriction patterns. Two inserts of approximately 2.2 kb were completely sequenced. The largest cDNA clone contained a continuous Open Reading Frame (ORF) starting 29 bp downstream of its

5' end. Comparison of the cDNA with the genomic clone revealed that the ORF is split into 9 exons coding for a predicted protein of 69.3 kDa.

The Ac insertion in *eir1-3* is located after codon 113 in exon 2 (Figure 2). The insertion is flanked by a perfect 8 bp direct repeat and probably results in a null allele of the affected gene. Results showed that *eir1-1* (as compared with the progenitor Columbia wild type) contains a transition mutation at the intron 5/exon 6 border that replaces the absolutely conserved G at splice position -1. (Brown, J.W.S., (1996) *Plant J. 10*: 771-780) The *eir1-1* mutation presumably results in a truncated EIR1 protein that would lack a conserved portion of the molecule (Figure 2).

To determine whether the cloned segment was the *EIR1* gene, *eir1-1* was transformed with the putative *EIR1* ORF and more than 2kb of upstream sequences. All five independent hygromycin-resistant transformants of *eir1-1* tested had a root growth phenotype typical of wild type. Therefore, the defects of the *eir1-1* mutant were complemented by the genomic fragment. No other large ORFs were present on the genomic fragment used in the transformation. Therefore, the open reading frame has been designated as the coding region of *EIR1*.

Isolation of *eir1-3* a new transposon-tagged allele of *EIR1*, permitted the cloning and characterization of both the mutant and wild type genes. Sequence analysis shows that *eir1-3* is an Ac insertion in the second of nine exons and *eir1-1* is a base substitution at a conserved splice site junction. Both of these mutations are likely to be null alleles because they should result in completely defective proteins. Expression of *EIR1* appears to be restricted to the root, which is consistent with the finding that all of the *eir1* mutant phenotypes, the most striking of which is gravitropism, affect the root and not other parts of the plant.

The amino acid sequence of *EIR1* is consistent with a role for this protein in transport of IAA. EIR1 is predicted to be an integral membrane protein. The presence of potential N-glycosylation sites and a potential N-terminal signal peptide indicates localization in the plasma membrane. EIR1 also has similarities to several membrane proteins involved in translocation of a variety of different substances across the plasma membrane. The transporters related to EIR1 are diverse in their substrate specificity and translocate amino acids, heavy metals, antibiotics, and dicarboxylic acids.

Perhaps the most compelling evidence that EIR1 plays a role in transport is that expression of *EIR1* in *S. cerevisiae* confers increased resistance to fluorinated analogues of indolic compounds. The resistance phenotypes are strongest in the *gef1* mutant, which has increased sensitivity to various compounds probably as a result of altered ion homeostasis (Gaxiola, R.A., *et al.*, (1998) *Proc. Natl. Acad. Sci. USA 95*: 4046-4050). Resistance to these indoles is completely dependent upon a functional *EIR1* gene product as neither *ClC-O* nor a mutated version of *EIR1* were capable of restoring yeast growth in the presence of fluorinated indolic compounds.

The EIR1 protein could prevent the inhibition of yeast by these compounds either by preventing their uptake or facilitating their efflux from the cytosol. The preferential localization of EIR1 in the plasma membrane of yeast is consistent with either of these mechanisms.

EXAMPLE 3

EIR1, a Highly Conserved Plant Gene Family with Similarities to Bacterial Transporters

Several lines of evidence suggest that *EIR1* belongs to a highly conserved gene family. Arabidopsis has several genes with considerable homology to *EIR*. In addition to several *Arabidopsis* ESTs (Genbank accession numbers: T04468, T43636, R84151, and Z38079), similar ORFs were found in database entries of the *Arabidopsis* Genome Initiative. Two close relatives dubbed *AEH1* and *AEH2* (for *Arabidopsis EIR1* Homologue) were located on clones T26J12 and MKQ4 on chromosome 1 and 5 respectively. These relatives probably account for the extra restriction fragments that hybridize to the EIR1 probe under conditions of high stringency. A related rice EST (accession number: D25054), which is derived from root-specific cDNA, was identified and sequenced (Figure 3). No other closely related sequences could be found outside the plant kingdom, suggesting that *EIR1* and its homologues represent a family of genes unique to higher plants.

Alignment of the deduced amino acid sequences of *EIR1*, *AEH1*, *AEH2*, and *REH1* (Rice *EIR1* Homologue) revealed that the regions of identity are restricted to the

N- and C-termini (Figure 3). Hydropathy plots and topology predictions identified 10 potential transmembrane domains shared by the 4 members of the gene family. The predicted EIR1 gene product comprises 647 amino acids and includes ten potential transmembrane domains flanking a central region enriched for hydrophilic amino acids. The predicted REH1 gene product comprises 595 amino acids and similarly comprises ten potential transmembrane domains flanking a central region which is predominantly hydrophilic. The transmembrane domains are located in the highly conserved portions of the proteins -- 5 at the N-terminus and 5 at the C-terminus (Figure 4).

The internal segments of the protein, though less conserved in sequence than the putative membrane spanning domains, exhibits a number of similarities. The central hydrophilic segments have a remarkably high content of serine and proline. EIR1 possesses a number of potential N-glycosylation sites, two of which are also found in REH1 and AEH1 (Figure 3). EIR1 has no ER-retention signal but does have a potential N-terminal signal peptide (von Heijne, G., (1986) *Nucleic Acids Res.14*: 4683-4690), which likely allows the protein to transit the secretory pathway to the plasma membrane. The open reading frame (ORF) of the EIR1 cDNA (SEQ ID NO.: 12) comprises nucleotides 19-1962 (Figure 6); the ORF of the REH1 cDNA (SEQ ID NO.: 13) comprises nucleotides 158-1945 (Figure 8).

The two hydrophobic portions of EIR1 show restricted similarity to a number of bacterial membrane proteins (Figure 4). The *mdcF* (U95087) protein is a potential malonate transporter from *Klebsiella pneumoniae* (Hoenke, S. *et al.*, (1997) *Eur. J. Biochem. 246*: 530-538), whereas *livM* (P22729) is involved in high affinity uptake of leucine into *Escherichia coli* (Adams, M.D., *et al.*, (1990) *J. Biol. Chem. 265*: 11436-11443).

Particularly noteworthy is the similarity of EIR1 to the class of efflux carriers that remove toxic compounds from the interior of the cell. For example, *E.coli arsB* (P37310) represents a part of the arsenic efflux system. (Diorio, C., *et al.*, (1995) *J. Bacteriol. 177*: 2050-2056). *sbmA* (X54153), another integral membrane protein of *E. coli*, has been shown to be necessary for uptake of the antibiotic Microcin 25 (Salomon, R.A. and R.N. Farias, (1995) *J. Bacteriol. 177*: 3323-3325). Portions of EIR1 show 35-40% similarity to these proteins. The finding that the N- and the C-

terminus of EIR1 exhibit similarities to the corresponding parts of bacterial transporters indicates that EIR1 is a membrane protein with a related function.

EXAMPLE 4

EIR1 Affects the Root-specific Response to Endogenous Ethylene

The reduced sensitivity of *eir1* roots to inhibition by ethylene suggested that *EIR1* might be a gene involved in regulation of ethylene responses specific to the root. In order to test this hypothesis, the response of the entire *eir1* mutant plant to endogenous ethylene was examined by constructing double mutants of *eir1* with *eto3* and *ctr1*. *eto3* causes overproduction of ethylene, giving rise to the typical triple response (the hypocotyl of plants germinated in the dark remains short, undergoes radial swelling and apical hook formation is exaggerated). Mutations in the Raf-like protein kinase *CTR1* phenocopy the ethylene-grown phenotype without elevating endogenous ethylene concentrations, suggesting that *CTR1* acts as a negative regulator of ethylene signal transduction (Kieber, J.J., *et al.*, (1993) *Cell 72*: 427-441).

The double mutants *eir1-3/eto3-1* and *eir1-3/ctr1-1* were germinated both in the dark and under constant illumination. Dark germinated plants still undergo the triple response, indicating that the *eir1* mutation has no influence on germination and early development of the aerial parts of the seedling . However, the inhibition of root elongation caused by *eto3* and *ctr1* mutations is considerably reduced in the double mutants.

These results suggest that reduced ethylene sensitivity of the *eir1* mutant is completely restricted to the root. Moreover, the phenotype is not caused by a block in biosynthesis or transport of ethylene because *eir1-3* bypasses the root phenotypes of *ctr1-1*, a mutation thought to be constitutive for the transduction of the ethylene signal.

Gravitropism, the curvature of the root in response to gravity, results from greater elongation of the upper side of the root than the lower side. Differential root elongation has been postulated to arise as the consequence of a gravity-induced auxin gradient with more auxin on the lower than the upper side (Kaufman, P.B., *et al.*,

(1995) In Plant Hormones Kluwer Academic Publishers, Dordrecht, Boston, London). The factors responsible for creating the auxin gradient are not known.

The simplest model to explain the phenotypes of the *eir1* mutant is that *EIR1* is required for efflux of auxin from the cells of the root tip into the elongation zone. If the root is oriented so that there is an increase in the auxin concentration on one side of the root tip, then EIR1 would pump auxin into the adjacent elongation zone with the concomitant inhibition of cell elongation. In *eir1* mutants the increased auxin in the lower portion of the root tip would fail to be transported into the elongation zone, and there would be no differential elongation. The predicted phenotypes of such a defect agree with those observed for an *eir1* mutation. The root should be agravitropic, and longer overall than an *EIR1* root. Furthermore, as described herein, increased levels of internal auxin should fail to inhibit the root or to induce root specific auxin inducible transcripts. The insensitivity of the *eir1* root to ethylene can be reconciled with the model if ethylene inhibits root growth by increasing the internal auxin concentrations (Suttle, J.C., (1991) *Plant Physiol. 96*: 875-880).

This model is also consistent with the response of *eir1* mutants to externally added auxins. If the *eir1* block were not in efflux, but rather in uptake of auxin, as has been proposed for *aux1* mutants (Bennett, M.J., *et al.*, (1996) *Science 273*: 948-50), then like the *aux1* mutants, the *eir1* mutants should be resistant to external auxin. However, the *eir1* mutants respond normally to external auxin. Root elongation is inhibited as in wild type, and induction of the *AtIAA2*-reporter construct appears to be unaffected.

EXAMPLE 5

EIR1 Expression is Localized to the Root

RNA-specific-PCR (RS-PCR) was used to analyze *EIR1* expression in the plant. Primers located on the 5' end of the *EIR1*-cDNA were used to amplify transcripts from reverse transcribed poly-A^+ RNA derived from roots, leaves, stems, flowers, and siliques. Primers for first strand cDNA synthesis were chimeric, having a 5' extension with no complementary sequences in the *Arabidopsis* genome. This sequence extension was used for subsequent PCR to avoid contamination. Genomic

DNA from ecotype Col-O served as a negative control. Results revealed a specific RS-PCR product in the root, but not in any other tissues. The root-specificity of *EIR1*-expression correlates well with the root-specific alterations detected in *eir1* mutants, suggesting that these defects are likely to be a consequence of the absence of *EIR1* function in the roots.

EXAMPLE 6

EIR1 Function is Required for Auxin Homeostasis in Root Cells

The involvement of EIR1 in root-specific auxin distribution was tested by analysis of the expression pattern of an auxin inducible gene, *AtIAA2*. The expression of *AtIAA2* has been shown to be strongly induced within a few minutes after exposure to auxin (Abel, S., *et al.*, (1996) *BioEssays. 18*: 647-654) The *AtIAA2* expression pattern was visualized using a reporter construct, *PIG4::GUS*, a transgene expressing β-glucuronidase under control of the AtIAA2-promoter. *AttIAA2* expression is strongest in the root meristem in wild type and *eir1-3*. When wild type is gravistimulated, expression of *AtIAA2* extends into the elongation and differentiation zone. Moreover, the expression is asymmetric with the lower portion of the elongation zone showing more intense staining than the upper. This asymmetric staining suggests that the lower portion of the elongation zone has elevated auxin levels as compared with the upper level. By contrast, reporter expression in *eir1-3* does not respond to the gravistimulus and remains restricted to the root tip.

The *eir1* root is known to be less sensitive to ethylene and to have an increased resistance to synthetic auxin transport inhibitors. These phenotypes could be explained if ethylene, like auxin transport inhibitors, interferes with tissue distribution of auxin. The effect of exogenous auxin on *PIG4::GUS* was assessed. Expression of *AtIAA2* has been shown to be strongly induced within a few minutes after exposure to auxin (Abel, S., *et al.*, (1995). *J Mol Biol. 251*: 533-49). In plants grown on regular medium, GUS staining is found in the root meristem and in the stele proximal to the root meristem. Addition of NAA (an auxin analogue) to the medium induces reporter gene expression in both the root meristem and elongation

zone of the root tip in wild type and the *eir1* mutant. Therefore, *eir1* mutants retain their ability to respond to exogenous auxin.

Plants (wild type and mutant) with the reporter responded quite differently to growth in ACC (the immediate biosynthetic precursor of ethylene) (1 μM ACC for 24 hours). In wild type, the entire elongation and differentiation zone shows considerable GUS staining upon ACC treatment. Furthermore, expression of GUS in the cell division zone appeared to be enhanced. In striking contrast, *eir1-3* mutant plant roots grown in ACC shows virtually no response in these tissues. Expression is restricted to the root tip at an intensity similar to that of plants grown in the absence of ACC.

The results with the auxin transport inhibitor TIBA are similar to those obtained with exogenous ACC. The reporter construct is induced in wild type but the mutant has a very reduced response. As auxin is the only known endogenous inducer of *AtIAA2* (Abel, S., *et al.*, (1996) *BioEssays. 18*: 647-654), ectopic expression of *AtIAA2* in wild-type roots treated with auxin transport inhibitors should be a consequence of elevated auxin concentrations in those cells that express the reporter. Unaltered *AtIAA2* expression in TIBA- and ACC-treated *eir1-3* roots suggests that auxin concentrations in cells of the root elongation zone remain unaffected when treated with these compounds.

The expression pattern of the auxin-inducible *AtIAA2::GUS* fusion in *eir1-3* is consistent with a block in auxin transport in the roots of this mutant. In wild type and *eir1-3* plants this reporter is expressed in root tips and at a low level in the younger parts of the vascular tissue. Wild type plants in the presence of ethylene, show increased expression of the reporter in the elongation zone, suggesting that these cells have an increased level of IAA.

The expression of the auxin-inducible reporter upon gravistimulation supports and extends these results. In wild type the auxin reporter is expressed asymmetrically, with more intense GUS-staining localized to the lower side of the elongation zone. This distribution is consistent with a model that proposes an inhibitory role for auxin in the regulation of root cell elongation and differential inhibition as the basis for gravitropism. Consistent with this interpretation, the

agravitropic *eir1-3* mutant grown under the same conditions fails to show differential staining or induction of the reporter in the elongation zone.

The failure of cells in the elongation zone of *eir1* roots to respond to IAA could be a consequence either of a failure to synthesize or to redistribute this growth regulator in response to ethylene. The effect of the *eir1* mutation on the root phenotype of the *alf1* mutant supports the redistribution hypothesis. The *alf1* mutation results in an approximately ten-fold increase in the endogenous concentration of IAA (Boerjan, W., *et al.*, (1995) *Plant Cell. 7*: 1405-1419). The high auxin level enhances the formation of lateral and adventitious roots but, also inhibits root elongation. Primary root growth in the *eir1 alf1* double mutant is not inhibited, showing that *eir1* suppresses the inhibitory effect of IAA on root elongation caused by the *alf1* mutation. However, *eir1* does not block the hyper-induction of lateral roots caused by *alf1*, showing that there are high levels of auxin in the root of the *eir1 alf1* double mutant.

These data are consistent with a model in which *EIR1* functions in auxin homeostasis in the root and auxin distribution in the root elongation zone. Two directions of auxin transport have been suggested for roots (Estelle, M., (1996) *Curr Biol. 6*: 1589-91): acropetal transport in the central cylinder from the base to the tip of the root and basipetal transport from the root tip to the elongation zone. If the inhibition of root growth in the *alf1-1* mutant results from the inhibition of cell expansion by excess auxin in the cells of the elongation zone, then the suppression of *alf1* by *eir1* is a consequence of *eir1's* defect in basipetal auxin transport into the elongation zone.

The root phenotype of *eto3* and *ctr1*, like that of the *alf1* mutant, is also suppressed by *eir1*. In both mutants the entire plant exhibits a strong ethylene response. *eto3* causes ethylene overproduction, whereas *ctr1* is probably a negative regulator of the ethylene response because *ctr*1 strains act as if they were in the presence of high ethylene although they do not have elevated ethylene concentrations (Kieber, J.J., *et al.*, (1993) *Cell. 72*: 427-441). The *eir1* mutant partially suppresses the *ctr1* phenotypes suggesting that EIR1 acts either downstream of ETO3 and CTR1 or in a pathway parallel to that in which ETO3 and CTR1 function (Roman, G., *et al.*, (1995) *Genetics. 139*: 1393-1409).

The decreased sensitivity of the *eir1* root to the inhibitory effects of ethylene as well as to the synthetic auxin transport inhibitors TIBA and NPA suggests a connection between auxin and ethylene. This behavior is similar to that of the *HOOKLESS1* (*HLS1*), mutants of *Arabidopsis* (Lehman, A., *et al.*, (1996) *Cell. 85*: 183-94). *HLS1* is thought to control bending in the apical tip of the hypocotyl because *hls1* mutants fail to form the apical hook during germination. Expression of the *HLS1* gene and enhanced hook formation are induced by treatment of plants with ethylene, which causes differential cell elongation. Remarkably, wild type seedlings grown in the presence of NPA have the same effect on apical hook formation and tissue distribution of auxin-induced genes as does the *hls1* mutant. Thus, auxin transport inhibitors phenocopy the *hls1* mutant, which is defective in the response of the apical hook to ethylene. These observations led to the speculation (Lehman, A., *et al.*, (1996) *Cell. 85*: 183-94) that an ethylene response gene may control differential cell growth by regulating auxin activity or distribution.

The growth characteristics of the *eir1* mutants also suggest a connection between auxin and ethylene. The *eir1* mutant root, like the apical hook of the *hls1* mutant is less sensitive to both exogenous and endogenous ethylene. Growth of wild type in the presence of auxin transport inhibitors blocks apical hook formation and the negative gravitropic response of the root. Moreover, like *hls1* the *eir1* roots are resistant to auxin transport inhibitors. In fact, this cross-resistance to both ethylene and auxin transport inhibitors is characteristic of mutants defective for auxin and ethylene responses (Fujita, H. and K. Syono, (1996) *Plant Cell Physiol. 37*: 1094-1101). This phenomenon probably represents an underlying mechanistic connection between the ethylene response and the auxin response, which is not yet understood.

EXAMPLE 7

eir1 Blocks the Inhibition of Root Growth Caused by High Endogenous Levels of Auxin

If *EIR1* is responsible for the redistribution of endogenous auxin, then the *eir1* mutation should block the defects in strains producing high levels of auxin. The effect of endogenous auxin was examined in *eir1-1 alf1-1* double mutants. The *alf1* mutation results in an enormously increased concentration of internal auxin, which leads to severe morphological alterations, which include the development of numerous short adventitious and lateral roots (Celenza, J.L., *et al.*, (1995) *Genes Dev. 9*: 2131-2142; Boerjan, W. *et al.*, (1995) *Plant Cell 7*: 1405-1419). The short root phenotype is caused by inhibition of cell elongation. The *eir1-1* mutation completely suppresses the short root phenotype caused by *alf1-1*, and retains the agravitropic phenotype, whereas the aerial portion of the *eir1 alf1* double mutant resembles *alf1*. These results suggest that in the eir1/alf1 double mutant elevated auxin levels do not reach the root elongation zone and that EIR1 is a tissue specific auxin transporter which is active in the root but not in the vascular tissue. Furthermore, the increased adventitious and lateral root formation, typical of *alf1* is not blocked by *eir1-3* suggesting that *eir1* represents a root tip-specific suppressor of the elevated auxin concentrations present in *alf1*.

EXAMPLE 8

Auxin transport in *Saccharomyces cerevisiae* Expressing *EIR1*

When auxin (IAA) is maintained under relatively acidic assay conditions (e.g., pH 4.0) it is protonated and thus capable of entering cells via diffusion across the plasma membrane. Once inside the cell the higher cytoplasmic pH acts as an ion-trap. IAAH dissociates and efflux of IAA-depends on anion transporters. We found that only in the absence of external carbon sources (compare Figures 10 A and B) there is a significant difference in the transport kinetics. Under these condiditions the ATP-requiring transporters of yeast are down as no new ATP is synthesized in the absence of an exogenous carbon source. However, yeast can maintain its intracellular (higher) pH for at least 30 minutes. This pH gradient is sufficient for EIR1-mediated ^{14}C-IAA transport as shown by the *gef1* and *gef1 EIR1* data (Figure

10B). Data resulting from the same experiment performed in the presence of the presence of the plasma-membrane specific protonophore CCCP demonstrates that under these under these conditions all differences in axuin transport activity between the EIR1-expressing and the control strain are gone (*gef1*+CCCP; *gef1 EIR1*+CCCP (Figure 10B)). Adding CCCP causes uptake of protons from the more acidic extracellular space into the cells. As a result the intracellular pH drops which gives rise to a protonation of IAA−. IAAH in turn can diffuse across the plasma membrane following a concentration gradient.

EXAMPLE 9

EIR1 in *Saccharomyces cerevisiae* Confers Increased Resistance to Flouroindolic Compounds

The growth of yeast strains that overexpress a plasmid borne *Arabidopsis EIR1* gene under the control of the *ADH1* promoter was analyzed. Wild type yeast strains are only slightly sensitive to fluorinated indolic compounds such as 5-DL-fluoro-tryptophan or 5-fluoro-indole, toxic analogues of potential precursors of IAA (Bartel, B., (1997) *Plant Mol. Biol. 48*: 51-66). However, strains, which carry the Δ*gef1* deletion (a mutant which alters ion homeostasis in yeast (Gaxiola, R.A., *et al.*, (1998) *Proc. Natl. Acad. Sci. USA 95*: 4046-4050)), are much more sensitive to 5-fluoro-indole, 5-fluoro-DL-tryptophan and 5-fluoro-indoleacetic acid. Remarkably, *gef1* strains that contain the *EIR1* gene were much more resistant to these indolic compounds than isogenic *gef1* strains with only a vector. The increased resistance conferred by EIR1 can also be observed in wild type, but the effect is more subtle because of the greater intrinsic resistance of strains with a functional *GEF1* gene.

Expression of the *EIR1* gene is required for this resistance because yeast strains containing a mutant form of the *EIR1* gene (a frameshift in the *EIR1* OF, plasmid pADE1-H) fail to show the increased resistance to fluoro-indoles. Moreover, this resistance is specific to these indolic compounds because strains carrying the *EIR1* gene are no more resistant than controls to fluconazole, another inhibitor of yeast growth. In addition, the increased resistance is not simply the consequence of expression of a foreign transporter in yeast. Expression of the

Torpedo marmorata chloride channel (*C1C-0*), which suppresses many of the *gef1* defects, failed to confer increased resistance to indolic compounds.

In order to localize the EIR1 protein in yeast, a functional, hemagglutinin (HA) epitope-tagged version of *EIR1* was introduced into *S. cerevisiae*. Examination of immunodecorated yeast cells using charge-coupled microscopy localized the most intense staining of EIR1 to the plasma membrane. This membrane localization is consistent with a role for EIR1 in excluding compounds from the cell and, thereby, preventing the toxicity of the indolic compounds.

EXAMPLE 10

Creation and Characterization of *EIR1* Alleles

Site-specific mutagenesis was performed in order to replace the conserved residue Ser97 of EIR1 with other amino acids. Three alleles were made: EIR1-S97G, EIR1-S97A and EIR1-S97E. Table 2 shows a comparison of the nucleotide and the deduced amino acid sequence of EIR1 and the three negative alleles proximal to Serine 97. The affected amino acid residue is typed in bold letters, alterations in the nucleotide sequence are indicated as lower case letters. Mutations were introduced by site-directed mutagenesis. No other alterations in the nucleotide sequences could be detected.

Table 2 EIR1 Ser97 mutants

EIR1	AGAGGAAGCCTA R G **S** L
EIR1-S97G	AGAGGAgGCCTA R G **G** L
EIR1-S97A	AGAGGAgcCCTA R G **A** L
EIR1-S97E	AGAGGAgagCTA R G **E** L

Expression of these alleles under control of the ADH-promoter (pADEI/S97G, pADEI/S97A and pADEI/S97E) was performed in diploid yeast strains, defective for

the *GEF1* gene (as described in Luschnig *et al.*, (1998) *Genes and Dev., 12(14):* 2175-2187). When testing these strains in a filter assay performed with either 5-fluoro-indole or 5-fluoro-indole acetic acid, these strains exhibit a hypersensitivity towards these toxic compounds. Moreover, these phenotypes appear to be conditional, as there are no growth differences detectable between strains expressing either EIR1 or one of the mutant alleles grown in regular medium. However, addition of 5-fluoro-indole (final concentration: 200 μM) to the liquid cultures, results in a reduced growth rate of yeast strains expressing the negative alleles.

A comparison of an HA-tagged version of EIR1 (pADEI-HA, Luschnig *et al.*, (1998) *Genes and Dev., 12(14):* 2175-2187) with an HA-tagged version of EIR1-S97G (pADEI/S97G-HA; a derivative of pADEI-HA in which an An *AgeI-PmlI* DNA fragment of pADEI-HA was replaced with the same fragment from pADEI/S97G carrying the Serine to Glycine substitution) revealed that EIR1-S97G no longer localizes to the plasma membrane, but is enriched in intracellular vesicle-like structures. A possible consequence of protein retention within the cell would be an increased concentration of the toxic, indolic compounds which, in turn, would explain the hypersensitivity of yeast strains, expressing the negative alleles. Increased intracellular concentrations of these compounds could be mediated by either binding of Flouroindolic to the mutant EIR1-protein or by increased uptake of the toxins into the vesicle-like structures.

Results showed that Serine 97 is of critical importance for correct targeting of EIR1 in yeast. Expression of three different alleles EIR1-S97G, -S97E, as well as -S97A, results in a reversion of (loss of) the 5 fluoro-indole resistance phenotype observed upon expression of the wild type EIR1 protein. *gef1* strains transformed with the different alleles under control of the ADH promoter were plated and tested for their growth in a filter assay. There was a dramatic increase in the zone of inhibition of the Flouroindolic for all of the gef1 transformants expressing one of the negative alleles. The growth delay caused by a replacement of Serine 97 does not interfere with yeast growth in the absence of Flouroindolic. The Growth curves of *gef1* strains transformed with either *EIR1* or one of the Ser97 mutants in Synthetic Complete medium (SC) (Figure 11A) or SC supplemented with 200 μM 5-fluoro-indole (Figure 11B) indicates that although growth in unsupplemented medium is not

affected by the mutations; growth in the presence of 5-fluoro-indole is severely reduced in all three mutant strains.

Immunodetection of hemagglutin (HA) epitope-tagged versions of EIR1-HA and EIR1/S97G-HA, performed according to the method of Example 9, revealed that EIR1/S97G-HA does not localize to the plasma membrane, as does EIR1-HA, rather it is enriched in intracellular vesicle-like structures.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. Isolated DNA encoding a protein involved in root-specific auxin transport, selected from the group consisting of:
   (a) DNA of SEQ ID NOS.: 11, 12 or 13 or portions thereof which encode a functional root-specific auxin transport protein;
   (b) DNA which, due to the degeneracy of the genetic code, encodes a protein having an amino acid sequence of SEQ ID NOS.:1 or 2;
   (c) DNA which hybridizes to DNA of (a) or (b) under high stringency conditions;
   (d) DNA which is sufficiently similar in sequence to DNA of (a), (b) or (c) to encode a root-specific protein involved in auxin transport;
   (e) DNA which encodes the amino acid sequence of SEQ ID NO.:2; and
   (f) isolated genomic DNA comprising DNA which encodes the amino acid sequence of SEQ ID NO.:2.

2. Isolated DNA selected from the group consisting of: SEQ ID NO.: 5, SEQ ID NO.:6, SEQ ID NO.:25 and SEQ ID NO.:26.

3. A genetically engineered plant comprising heterologous DNA encoding a root- specific protein involved in auxin transport, wherein the genetically altered plant is more resistant to an herbicidal composition which comprises at least one chemical compound which is auxin, an auxin derivative, an auxin analogue, or an auxin transport inhibitor.

4. The genetically engineered plant of Claim 3, wherein the heterologous DNA is selected from the group consisting of:
   (a) DNA of SEQ ID NOS.: 11, 12 or 13 or portions thereof which encode a functional root-specific auxin transport protein;
   (b) DNA which, due to the degeneracy of the genetic code, encodes a protein having an amino acid sequence of SEQ ID NOS.: 1 or 2;

(c) DNA which hybridizes to DNA of (a) or (b) under high stringency conditions;

(d) DNA which, is sufficiently similar in sequence to DNA of (a), (b) or (c) to encode a root-specific protein involved in auxin transport;

(e) DNA which encodes the amino acid sequence of SEQ ID NO.:2; and

(f) isolated genomic DNA comprising DNA which encodes the amino acid sequence of SEQ ID NO.:2.

5. The genetically engineered plant of Claim 4 which is a crop plant or a flowering plant.

6. A method of enhancing transport of an auxin derivative or an auxin analogue in plant roots comprising introducing into a plant part, including a seed, a gene which encodes a root-specific auxin transport protein and growing the plant part or seed under conditions appropriate for production of a plant, wherein the roots of the resulting plant contain the gene and express the encoded protein in sufficient quantity to enhance transport of auxin.

7. A genetically engineered plant comprising a heterologous DNA encoding a root-specific protein involved in auxin transport, wherein the genetically engineered plant exhibits altered auxin homeostasis relative to the auxin homeostasis of a wild type plant.

8. The genetically engineered plant according to Claim 7 wherein the heterologous DNA comprises isolated DNA selected from the group consisting of:

(a) DNA of SEQ ID NOS.: 11, 12 or 13 or portions thereof which encode a functional root-specific auxin transport protein;

(b) DNA which, due to the degeneracy of the genetic code, encodes a protein having an amino acid sequence of SEQ ID NOS.:1 or 2;

(c) DNA which hybridizes to DNA of (a) or (b) under high stringency conditions;

(d) DNA which, is sufficiently similar in sequence to DNA of (a), (b) or (c) to encode a root-specific protein involved in auxin transport;

(e) DNA which encodes the amino acid sequence of SEQ ID NO.:2; and

(f) isolated genomic DNA comprising DNA which encodes the amino acid sequence of SEQ ID NO.:2.

9. The genetically engineered plant according to Claim 8, wherein the altered auxin homeostasis results in an increased number of lateral or adventitious roots.

10. A genetically engineered plant comprising isolated DNA according to Claim 1 wherein the plant is characterized by an increased auxin transport rate relative to the auxin transport rate of a corresponding wild type plant.

11. A method of identifying an allele of EIR1 which confers altered auxin-mediated responses in a plant, comprising the steps of:

(a) introducing a mutated EIR1 nucleic acid into yeast cells under conditions in which the DNA is expressed,thereby producing transformed yeast cells;

(b) contacting the transformed yeast cells of (a) with a fluorinated indolic compound under assay conditions which favor diffusion of the compound into the transformed yeast cells;

(c) determining the growth phenotype of the cells of (b); and

(d) comparing the growth phenotype of the transformed yeast cells to the growth phenotype of wild type cells

wherein detection of an altered growth phenotype in the transformed cells relative to the growth phenotype in wild-type cells indicates that the mutant EIR1 nucleic acid is an allele of EIR1 which confers altered auxin-mediated responses in a plant.

12. The method of Claim 11 in which the yeast cell is a diploid yeast strain defective for the GEF1 gene.

13. The method of Claim 12 wherein the fluorinated indolic compound is selected from the group consisting of: 5-DL-fluoro-tryptophan, 5-fluoro-indole and 5-fluoro-indolacetic acid.

14. The method of Claim 12 wherein the altered growth phenotype associated with expression of the mutated EIR1 nucleotide sequence comprises tolerance to concentrations of a fluorinated indolic compound which are toxic to wild type cells.

15. The method of Claim 12 wherein the altered growth phenotype associated with expression of the mutated EIR1 nucleotide sequence comprises increased
sensitivity to concentrations of a fluorinated indolic compound which are not toxic to wild type cells.

16. A transgenic plant comprising an allele of EIR1 DNA identified by the method of Claim 12.

17. Isolated or recombinantly produced root-specific protein involved in auxin transport and alleleic variants thereof.

18. The protein of Claim 17 comprising an amino acid sequence selected from the group consistig of: SEQ ID NO.: 1 and SEQ ID NO.: 2.

19. An expression vector comprising DNA selected from the group consisting of: SEQ ID NO.: 11, SEQ ID NO.: 12 and SEQ ID NO.: 13.

20. A method of producing a transgenic plant characterized by altered auxin homeostasis comprising the steps of:

(a) introducing DNA encoding a root-specific auxin transport carrier protein into a plant cell under conditions in which the DNA is expressed, thereby producing a transformed plant cell; and

(b) producing a transgenic plant from the transformed plant cell.

21. The method of Claim 20, wherein the DNA encoding a root-specific auxin transport carrier protein is selected from the group consisting of:

(a) DNA of SEQ ID NO.: 11, 12 or 13 or portions thereof which encode a functional root-specific auxin transport protein;

(b) DNA which, due to the degeneracy of the genetic code, encodes a protein having an amino acid sequence of SEQ ID NOS.:1 or 2;

(c) DNA which hybridizes to DNA of (a) or (b) under high stringency conditions;

(d) DNA which, is sufficiently similar in sequence to DNA of (a), (b) or (c) to encode a root-specific protein involved in auxin transport;

(e) DNA which encodes the amino acid sequence of SEQ ID NO.:2; and

(f) isolated genomic DNA comprising DNA which encodes the amino acid sequence of SEQ ID NO.:2.

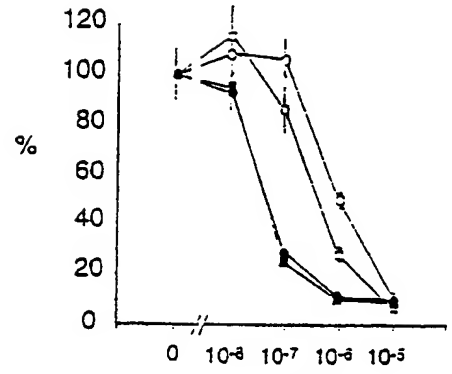


FIG. 1A

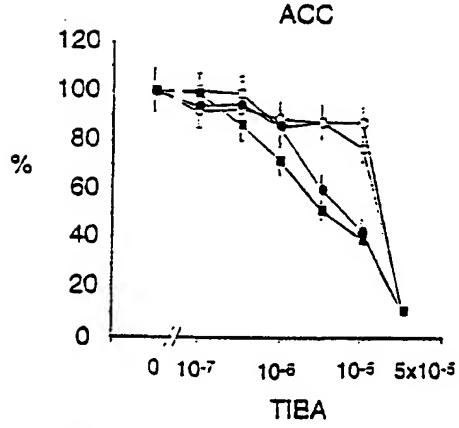


FIG. 1B

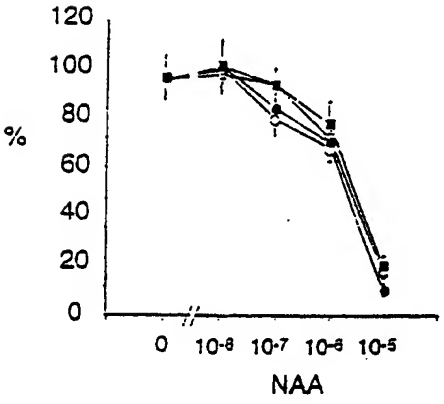
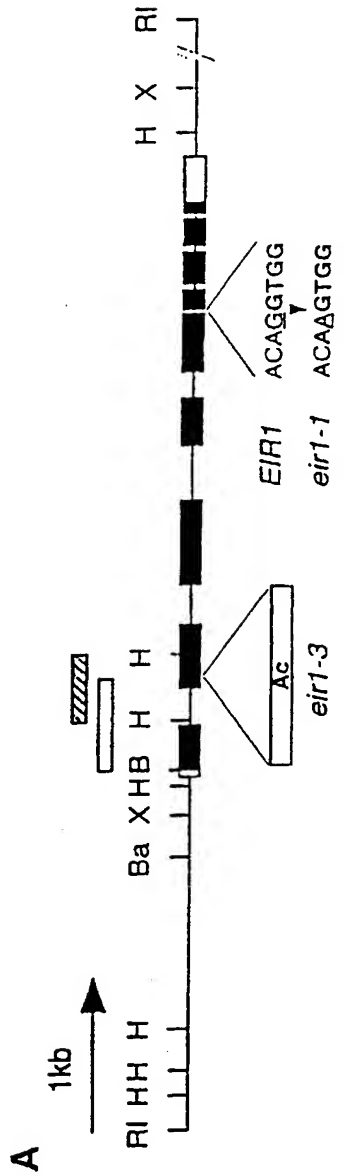


FIG. 1C


A
1kb
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Ba X H B H H
H X RI
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ACAΔGTGG
EIR1
eir1-1
Ac
eir1-3

FIG. 2

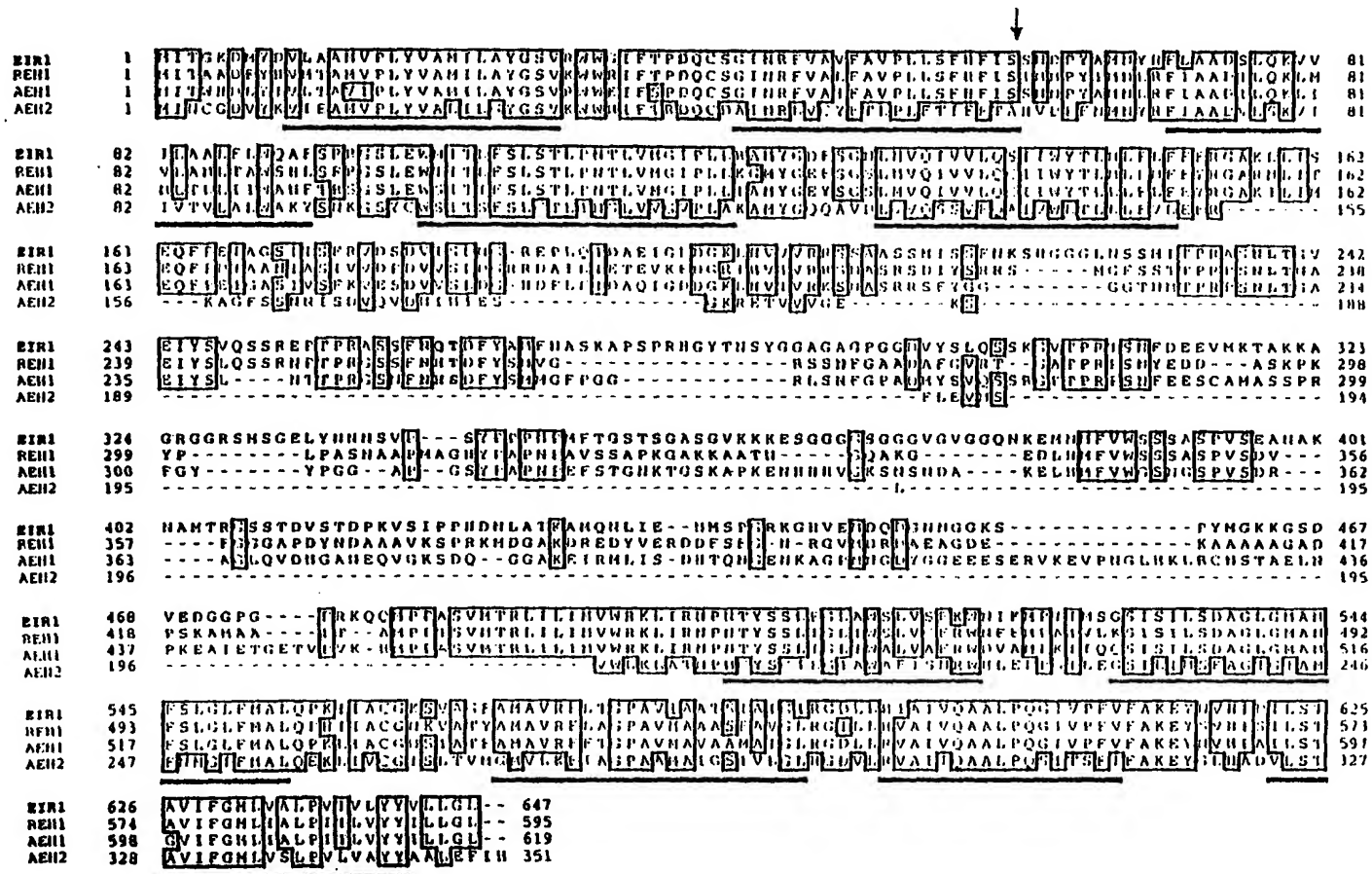


FIG. 3

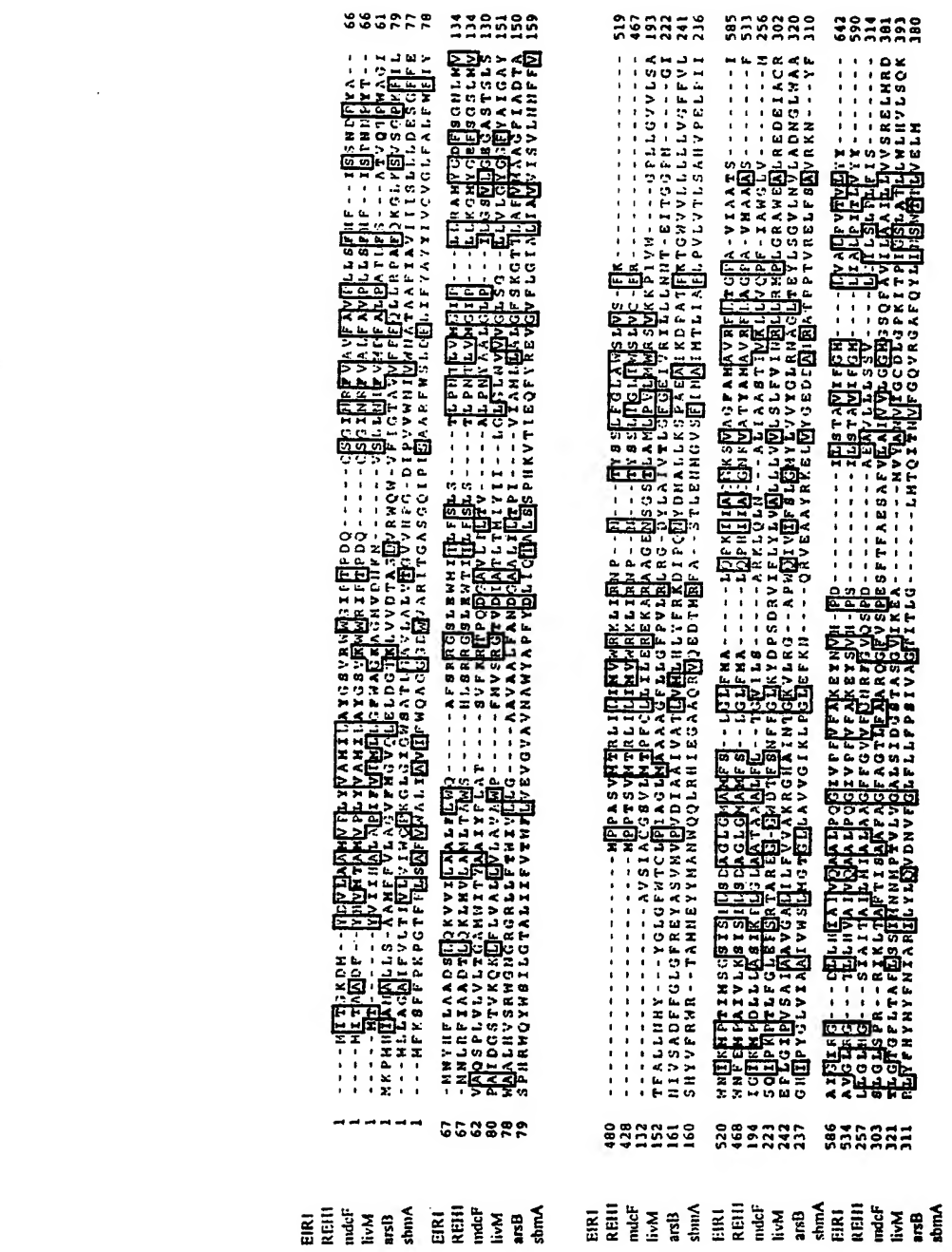


FIG. 4

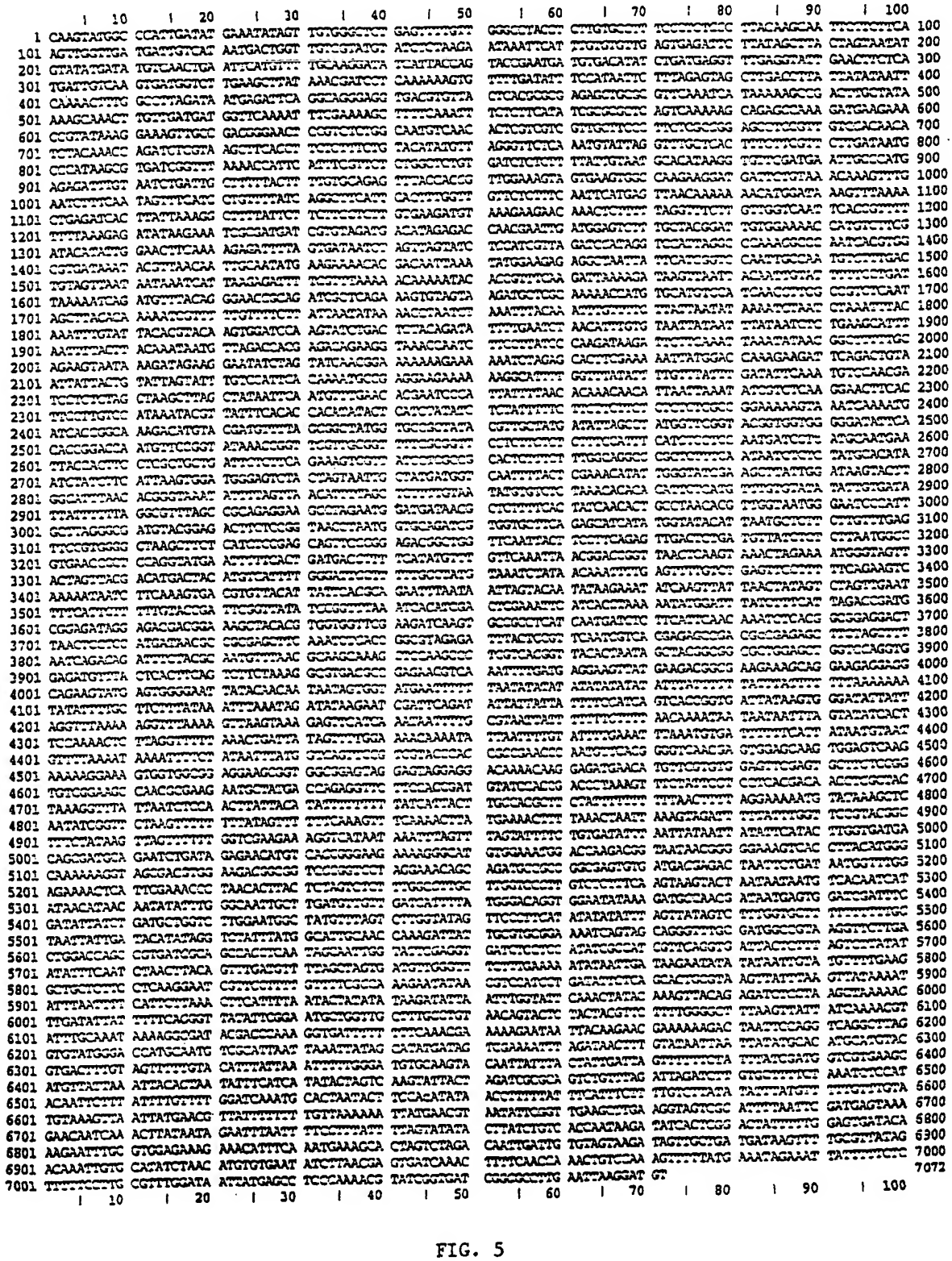


FIG. 5

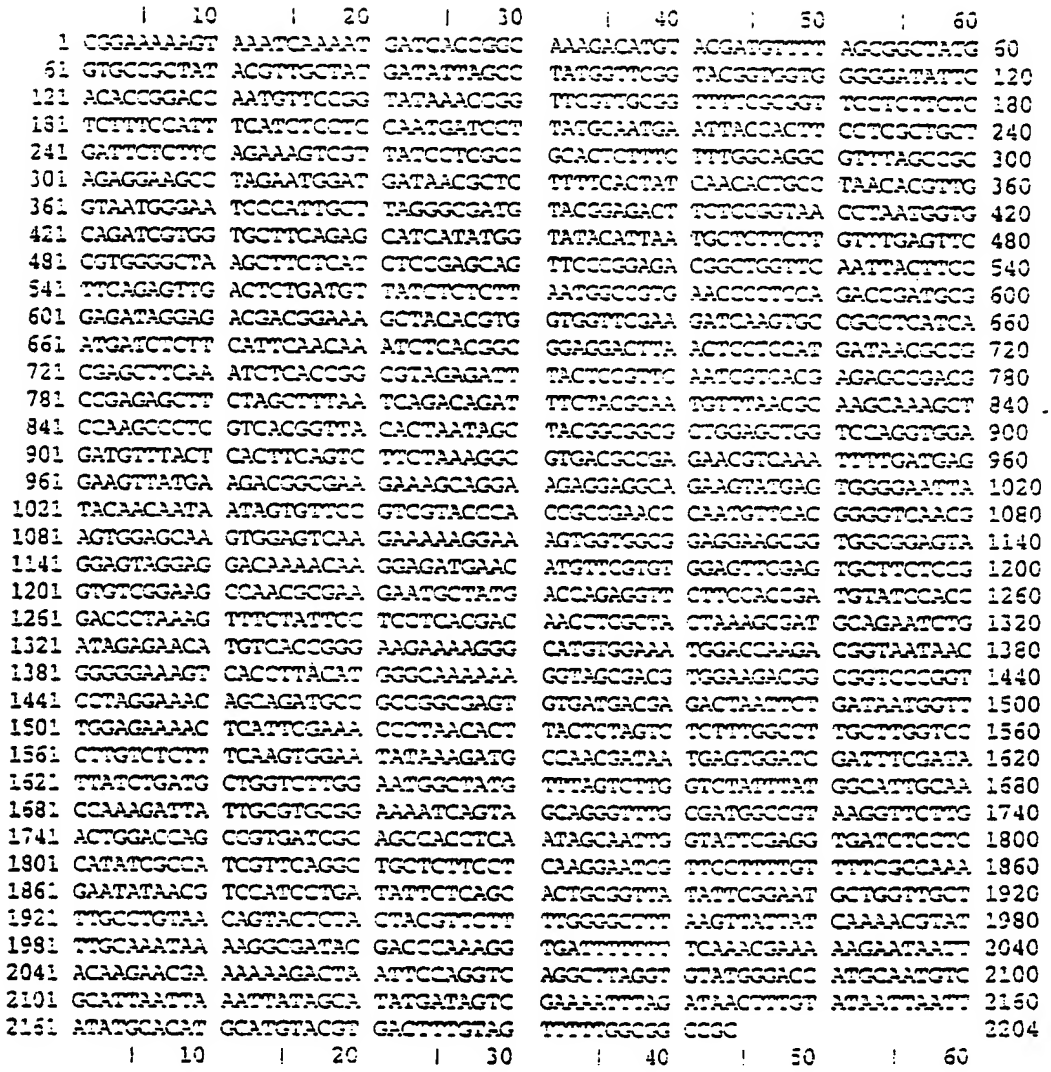
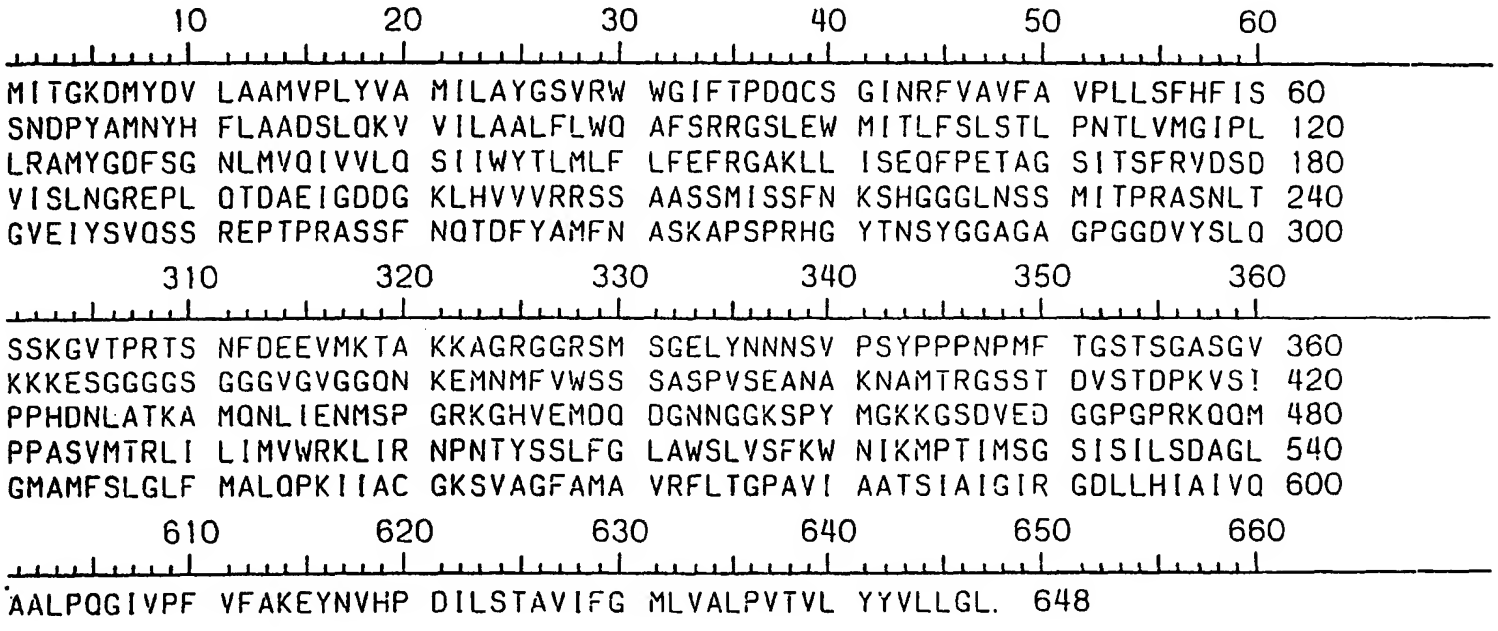


FIG. 6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LRAMYGDFSG NLMVQIVVLQ SIIWYTLMLF LFEFRGAKLL ISEQFPETAG SITSFRVDSD 180
VISLNGREPL QTDAEIGDDG KLHVVVRRSS AASSMISSFN KSHGGGLNSS MITPRASNLT 240
GVEIYSVQSS REPTPRASSF NQTDFYAMFN ASKAPSPRHG YTNSYGGAGA GPGGDVYSLQ 300
310 320 330 340 350 360
SSKGVTPRTS NFDEEVMKTA KKAGRGGRSM SGELYNNNSV PSYPPPNPMF TGSTSGASGV 360
KKKESGGGGS GGGVGVGGQN KEMNMFVWSS SASPVSEANA KNAMTRGSST DVSTDPKVSI 420
PPHDNLATKA MQNLIENMSP GRKGHVEMDQ DGNNGGKSPY MGKKGSDVED GGPGPRKQQM 480
PPASVMTRLI LIMVWRKLIR NPNTYSSLFG LAWSLVSFKW NIKMPTIMSG SISILSDAGL 540
GMAMFSLGLF MALQPKIIAC GKSVAGFAMA VRFLTGPAVI AATSIAIGIR GDLLHIAIVQ 600
610 620 630 640 650 660
AALPQGIVPF VFAKEYNVHP DILSTAVIFG MLVALPVTVL YYVLLGL. 648

FIG. 7

```
        |   10      |   20      |   30      |   40      |   50      |   60
   1 GTCGACCGAC GCGTCCGCAC ACCCAATCAA ATGCTCCTCC TCCGCCGCCT CCTCTCGCTG 60
  61 AGCTGAGCTG AGCTGTGAAA TAGTGCCACC GAGTGAGCGC TGAGCTGAGC TCGAAGCAAG 120
 121 AGAGGAAGAG GAGGAGGAGC GAAGAGGGGG GGCGAAGATG ATTACGGCGG CGGACTTCTA 180
 181 CCACGTGATG ACGGCGATGG TGCCGTTGTA CGTGGCGATG ATACTGGCGT ACGGGTCGGT 240
 241 GAAGTGGTGG CGCATCTTCA CGCCGGACCA GTGCTCCGGG ATCAACCGCT TCGTGGCGCT 300
 301 CTTCGCCGTG CCGCTGCTGT CGTTCCACTT CATCTCCACC AACAACCCGT ACACGATGAA 360
 361 CCTCCGGTTC ATCGCCGCCG ACACGCTGCA GAAGCTGATG GTGCTGGCCA TGCTCACGGC 420
 421 GTGGAGCCAC CTCAGCCGCC GGGGGAGCCT CGAGTGGACC ATCACGCTCT TCTCCTCTC 480
 481 CACGCTGCCC AACACGCTCG TCATGGGGAT CCCTTTGCTC AAGGGCATGT ACGGGGAGTT 540
 541 CTCCGGCAGC CTCATGGTGC AGATCGTCGT GCTGCAGTGC ATCACTCTGG TACACGCTCAT 600
 601 GCTCTTCATG TTCGAGTACC GCGGCGCCCG GATGCTCATC ACCGAGCAGT TCCCGGACAC 660
 661 CGCCGCCAAC ATCGCCTCCA TCGTCGTCGA CCCGGACGTC GTGTCGCTGG ACGGCAGGAG 720
 721 GGACGCCATC GAGACGGAGA CGGAGGTGAA GGAGGACGGC AGGATACACG TCACCGTGCG 780
 781 CCGCTCCAAC GCGTCTCGCT CGGACATCTA CTCCGCCGC TCCATGGGCT TCTCCAGCAC 840
 841 CACGCCGCGG CCGAGCAACC TCACCAACGC CGAGATCTAC TCGCTGCAGT CGTCGCGGAA 900
 901 CCCGACGCCG AGGGGTTCAA GCTTCAACCA CACCGACTTC TACTCGATGG TGGGCCGCAG 960
 961 CTCCAACTTC GGCGCGGCCG ACGCGTTCGG CGTCCGCACC GGCGCCACGC CGCGCCCGTC 1020
1021 CAACTACGAG GACGACGCGT CCAAGCCCAA GTACCCGCTC CCGGCGTCGA ATGCGGCGCC 1080
1081 CATGGCGGGC CACTACCCGG CGCCGAACCC GGCCGTGTCG TCGGCGCCCA AGGGCGCCAA 1140
1141 GAAGGCGGCC ACGAACGGGC AGGCCAAGGG CGAGGACCTC CACATGTTCG TCTGGAGCTC 1200
1201 CAGCGCGTCG CCCGTGTCCG ACGTCTTCGG CGGCGGCGCC CCAGACTACA ACGACGCCGC 1260
1261 GGCAGTCAAG TCCCCCCGCA AAATGGATGG AGCGAAGGAC AGGGAGGACT ACGTGGAGCG 1320
1321 GGACGATTTC AGCTTCGGGA ACAGGGGCGT CATGGACAGG GACGCGGAGG CAGGGGACGA 1380
1381 GAAGGCGGCG GCGGCGGCGG GCGCCGACCC CAGCAAGGCC ATGGCGGCGC CGACGGCGAT 1440
1441 GCCGCCGACG AGCGTGATGA CCCGCCTCAT CCTGATCATG GTGTGGCGCA AGCTCATCCG 1500
1501 CAACCCGAAC ACCTACTCCA GCCTCATCGG CCTCATCTGG TCCCTCGTCT GCTTCAGGTG 1560
1561 GAACTTCGAG ATGCCGGCCA TCGTCCTGAA ATCCATCTCG ATCCTGTCGG ACGCGGGGCT 1620
1621 CGGCATGGCC ATGTTCAGTC TCGGTCTGTT CATGGCGCTG CAGCCGCACA TCATCGCGTG 1680
1681 CGGGAACAAG GTGGCGACGT ACGCCATGGC GGTGCGGTTC CTGGCCGGGC CGGCCGTGAT 1740
1741 GGCGGCGGCG TCCTTCGCCG TCGGACTCCG TGGCACGCTC CTGCAGTTCG CCATTGTCCA 1800
1801 GGCAGCTCTG CCCCAGGGCA TTGTCCCCTT CGTCTTCGCC AAGGAGTACA GCGTGCACCC 1860
1861 TAGCATTCTC AGCACAGCTG TCATCTTTGG CATGCTCATC GCCTTGCCTA TCACCTTCGT 1920
1921 CTACTACATC TTGCTTGGGC TGTAATCGAG TTGCATGCAC GTAAATTCCT GCTCCTGACA 1980
1981 ACCAGCCATG TTAAGAAGAG GGGAGAAGAA GACAGAGCTG GTACACTGTT TGCAAAGTCA 2040
2041 GGACTCTTTG ATTTTTCTTT TCTTTTCTGT ATTTCTTGAA GTAGAATTTG GGAGGAGGGG 2100
2101 GATTGGAAGG GAGTCAAACG AGTCAAGGGG AGGACAGGAT GGTAGCTAGC TTAGCTAGGA 2160
2161 CAATGGTGAG TCACAAAAGA GCACCAAAAG CAAGTACAAG TACAAAGCTT GGGGGGACAC 2220
2221 AGGATCCAGT TCAGGTCACA GAAACGGTTC GGTTTTGGGA GGGGATTGTG GGAGTTTTGG 2280
2281 TTGGCTGCGC TGCGCTGACC CTTGTAAAAC GGACGCCGAT TCTGACAAGA GATCGACCTT 2340
2341 GTTTTAAAAA AAAAAAAAAA AAAAGGGCGG CCGC                           2374
        |   10      |   20      |   30      |   40      |   50      |   60
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FIG. 8

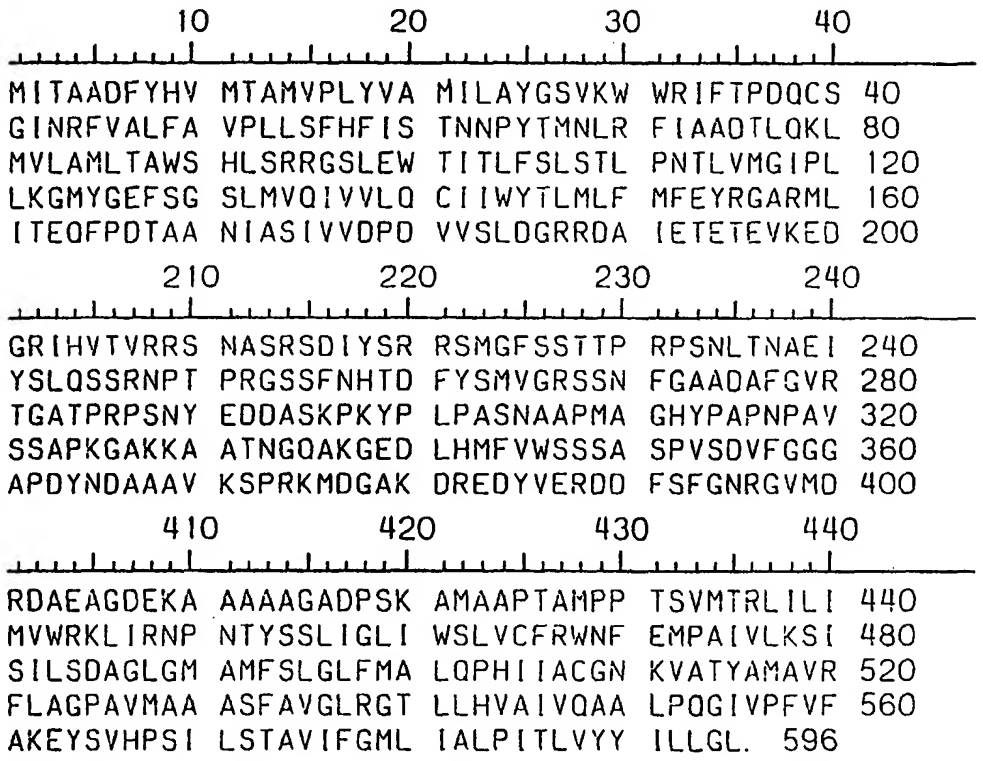
10 20 30 40
MITAADFYHV MTAMVPLYVA MILAYGSVKW WRIFTPDQCS 40
GINRFVALFA VPLLSFHFIS TNNPYTMNLR FIAAQTLQKL 80
MVLAMLTAWS HLSRRGSLEW TITLFSLSTL PNTLVMGIPL 120
LKGMYGEFSG SLMVQIVVLQ CIIWYTLMLF MFEYRGARML 160
ITEQFPDTAA NIASIVVDPD VVSLQGRRDA IETETEVKED 200
210 220 230 240
GRIHVTVRRS NASRSDIYSR RSMGFSSTTP RPSNLTNAEI 240
YSLQSSRNPT PRGSSFNHTD FYSMVGRSSN FGAADAFGVR 280
TGATPRPSNY EDDASKPKYP LPASNAAPMA GHYPAPNPAV 320
SSAPKGAKKA ATNGQAKGED LHMFVWSSSA SPVSDVFGGG 360
APDYNDAAAV KSPRKMDGAK DREDYVERDD FSFGNRGVMD 400
410 420 430 440
RDAEAGDEKA AAAAGADPSK AMAAPTAMPP TSVMTRLILI 440
MVWRKLIRNP NTYSSLIGLI WSLVCFRWNF EMPAIVLKSI 480
SILSDAGLGM AMFSLGLFMA LQPHIIACGN KVATYAMAVR 520
FLAGPAVMAA ASFAVGLRGT LLHVAIVQAA LPQGIVPFVF 560
AKEYSVHPSI LSTAVIFGML IALPITLVYY ILLGL. 596

FIG. 9

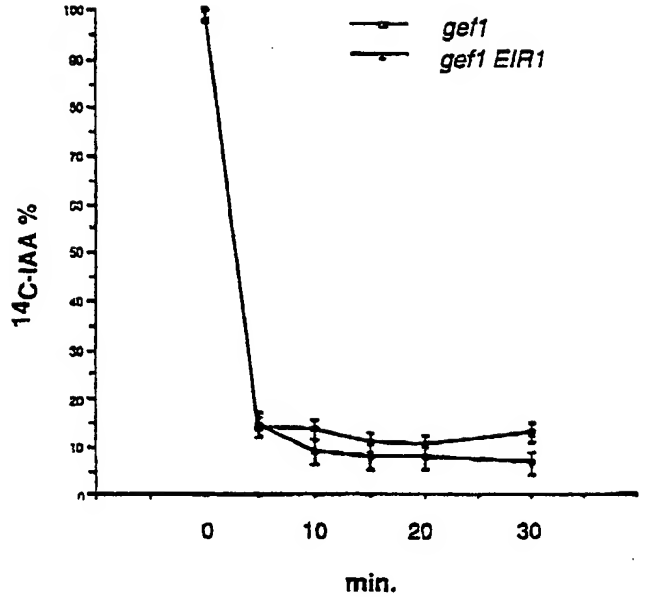

FIG. 10A

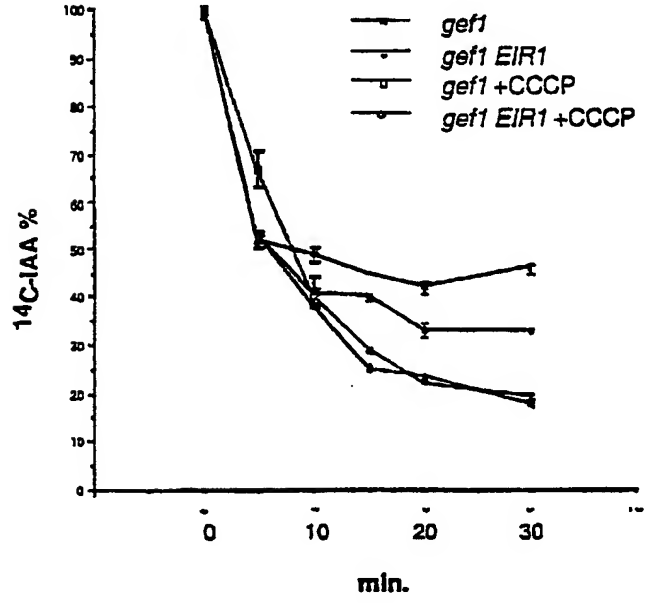

FIG. 10B

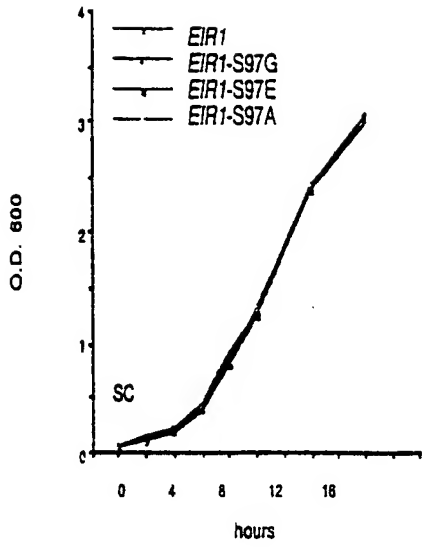

FIG. 11A

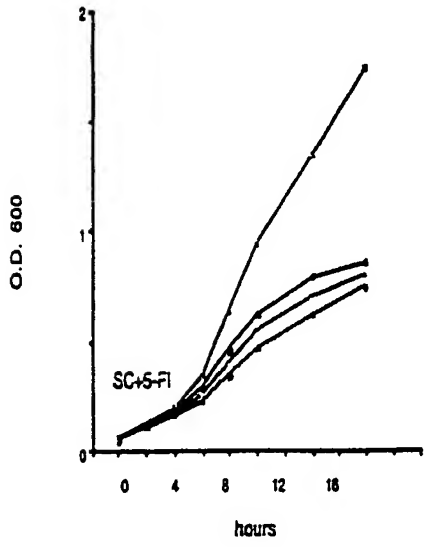

FIG. 11B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/12277

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 A01H5/00 C07K14/415

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 49810 A (MAX PLANCK GESELLSCHAFT ✓ ;GAELWEILER LEO (DE); PALME KLAUS (DE); WI) 31 December 1997 (1997-12-31) the whole document ---	1-11,17, 20,21
P,X	LUSCHNIG C ET AL.: "EIR1, a root-specific ✓ protein involved in auxin transport, is required for gravitropism in Arabidopsis thaliana" GENES AND DEVELOPMENT, vol. 12, no. 14, 15 July 1998 (1998-07-15), pages 2175-2187, XP002116368 the whole document ---	1-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 September 1999	15/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Bilang, J

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/12277

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MÜLLER A ET AL.: "AtPIN2 defines a locus of Arabidopsis for root gravitropism control" EMBO JOURNAL, vol. 17, no. 23, 1 December 1998 (1998-12-01), pages 6903-6911, XP002116369 the whole document	1-11,17, 20,21
P,X	UTSUNO K ET AL.: "AGR, an Agravitropic locus of Arabidopsis thaliana, Encodes a Novel Membrane-Protein Family Member" PLANT AND CELL PHYSIOLOGY, vol. 39, no. 10, October 1998 (1998-10), pages 1111-1118, XP002116370 the whole document	1-11,17, 20,21
A	ROMAN G ET AL.: "Genetic Analysis of Ethylene Signal Transduction in Arabidopsis thaliana: Five Novel Mutant Loci Integrated into a Stress REsponse Pathway" GENETICS, vol. 139, no. 3, March 1995 (1995-03), pages 1393-1409, XP002116371 page 1397, column 1, paragraph 2 - column 2, paragraph 1	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/12277

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9749810 A	31-12-1997	EP 0814161 A	29-12-1997
		AU 3539597 A	14-01-1998
		CA 2259125 A	31-12-1997